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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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AUGUST, 1971

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INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers, preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

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THE BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

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I. TRUSTEES

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL] HENRY B. PIERCE,
Secretary of the Commonwealth

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 12, 1966)

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and nine Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-six Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed.

(B) Trustees *ex officio*, who shall be the Chairman, the Director, the Treasurer, and the Clerk.

C. Trustees *Emeriti*, who shall be elected from present or former Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that *Trustees Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation They shall elect a President of the Corporation shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the officers. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED AT TRUSTEES' MEETINGS

EXECUTIVE COMMITTEE

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of not more than ten members including the *ex officio* members who shall be the Chairman of the Board of Trustees, President, Director and Treasurer; six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term. (August 11, 1967).

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine. (August 12, 1966)

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or laws. (August 16, 1963)

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its action shall be reported to the Board of Trustees. (August 16, 1963)

II. RESOLVED:

The elected members of the Executive Committee shall be constituted as a standing "Committee for the Nomination of Officers," responsible for making nominations at the Annual Meeting of the Corporation and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk). (August 16, 1963)

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

Gentlemen:

By convention, annual reports deal largely, if not entirely, with past and already cold events. In these reports I will, from time to time, depart from convention to speak of ambitions for the future, of plans being formulated, and of new undertakings. Undeniably, the future of the Laboratory can be predicted only to a limited degree, for opportunism often plays a large role in advances in science, and today, more than ever before, we must be alert to advantageous alterations in course. However the Laboratory, in pace with the scientific community as a whole, has entered a period of self-examination, a search for a new perspective and sense of purpose, recognizing that, in S. L. Fawcett's words, ". . . man's progress depends upon a balanced effort resulting in both new knowledge and innovative uses of this knowledge." In this period, it is especially fitting that ambitions and plans be discussed as fully as possible.

I would begin by reiterating my observations at the Annual Meeting, August 14, 1970.

The year 1970 was at once a difficult, yet reassuring year for me. I arrived in mid-June full of concern about the Laboratory. It had been a hard winter. Although an aura of success enveloped the Laboratory, with new buildings completed and in progress, and a summer program of international fame, our financial situation continued to worsen, and it was only through the determined efforts of our Chairman and Treasurer and the economies effected by Mr. Smith and his staff that the downward trend was slowed—although by no means halted or reversed. There were other distress signals. As we heard from the Committee on Research Space, in recent years the number of applications for laboratories has been very close to the number finally accepted. Less than five per cent of those applying have been denied space. In one sense this figure speaks to the effectiveness of Burr Steinbach, who by cajolery, compromise, and measures known only to him, has kept many applicants happy (or reasonably so) with less space than they desired, thus accommodating more investigators. However we would have hoped that his job—now mine—would have been even more difficult. We need more competition for our space.

Yet I began by saying that I was also reassured. It is not that the problems miraculously disappeared. Far from it; what reassured me was the realization that the members of the Corporation and other scientists in residence shared my concern and were willing to take steps to meet the problems, even though in doing so they added to burdens they were already bearing in their home institutions. It is for all of us a frustrating period, a time of national indecision. Not only do we fail to gauge the future, we find it difficult even to understand the present.

I am reminded of the remark attributed to Abbé Sieyès, who when asked what he did during the French Revolution, replied, "I survived." The Laboratory must do more than "survive." It must not be becalmed, for there is nothing more dangerous than to be paralyzed between apprehension and action.

At their meeting in February, 1970, the Trustees and officers resolved that the Laboratory had to move forward. That resolve was translated into action during the year. It should be emphasized that although the Laboratory's plans for the future are an outgrowth and elaboration of ideas developed over the past decade by the Trustees, working with the two previous directors, P. B. Armstrong and H. B. Steinbach, at least sixty members of the Corporation and resident scientists played an active role in their formulation during 1970.

In the spring of 1970, a draft proposal was prepared by Steinbach and Ebert, then Director and Director-designate respectively, in consultation with the Chairman of the Board of Trustees, Mr. Gerard Swope, Jr., and the General Manager, Mr. Homer P. Smith. This draft was prepared as a basis for further discussion and planning. It was circulated to the Trustees, and discussed extensively at a full scale meeting of the Board on July 2, 1970.

The Trustees approved the formation of a representative Planning Committee, which was established on July 7. Subsequently, the Planning Committee itself initiated three sub-groups. The composition of the Committee and Sub-Committees follows:

> *Planning Committee:* H. O. Halvorson and J. W. Hastings, *Co-Chairmen*, John Buck, F. D. Carlson, Sears Crowell, R. T. Hinegardner, C. L. Prosser, S. M. Rose, H. W. Siegelman and W. S. Vincent.
>
> *Sub-Committee on Visiting Scholars:* H. O. Halvorson, *Chairman*, Martha Baylor, M.V.L. Bennett, Philip Grant, Hugh Huxley, Alexander Keynan, and Cyrus Levinthal.
>
> *Sub-Committee on Education:* W. S. Vincent, *Chairman*, James F. Case, Frank Child, Arthur Humes, Jonathan Green, and Allan Scott.
>
> *Sub-Committee on the MBL as a Conference Center:* Ralph Hinegardner, *Chairman*, A. M. Clark, Cyrus Levinthal, Robert Loftfield, and Maurice Sussman.

The Planning Committee held nine meetings between July 10 and August 21. The Sub-Committee on Education met seven times, that on Visiting Scholars, four times. The Conference Sub-Committee collected written recommendations from its members. On July 28, the Planning Committee solicited the help of all Corporation Members and Individuals registered at the MBL by letter. In response to this request, and to a later request by the Director, there were at least 50 written statements, and innumerable conversations. In addition to input from its Sub-Committees, the Planning Committee drew extensively upon the reports of several of the Standing Committees, especially the Supply Department, Research Services, Buildings and Grounds and Library Committees. Ideas were also contributed by members of the Systematics-Ecology and Boston University Marine Programs. The Committee's report was submitted to the Director on August 21.

Concurrently during July and August, a second major Committee was in operation. Headed by John Dowling, the Neuroscience Committee was charged with evaluating the Laboratory's special needs in this fast-moving field:

> *Neurosciences Committee:* John Dowling, *Chairman*, W. J. Adelman, M. V. L. Bennett, J. F. Case, Timothy Goldsmith and E. O. Wilson.

Penultimate draft proposals were prepared during October 1970 for review by the Executive Committee and those who had played key roles in the studies (about 50 individuals). Their further comments were incorporated in two proposals, one submitted on January 20, 1971 to the Alfred P. Sloan Foundation for support of a large part of the Laboratory's teaching programs in the neurosciences, and the second submitted on May 12, 1971 to the National Science Foundation.

The proposals: (1) neuroscience training

Recognizing that the Laboratory holds a unique position of strength in research in the neurosciences, we will make a sustained effort to increase our highly promising training programs in that field—programs that are now underway only by dint of sacrifice on the part of Drs. Adelman, Bennett, Dowling and their colleagues, and the generosity and good will of companies and individuals who have loaned equipment, and through modest support provided by the Laboratory. One of our immediate large goals should be to provide facilities for the Neurobiology course in the Loeb Teaching Building, and to provide improved quarters for Excitable Membrane Biophysics and Physiology. To these ends we are seeking major new funding from foundations. I should note that the projected new quarters for the Neurobiology course would serve not only our summer programs, but eventually our winter programs as well.

I would emphasize that the Neuroscience Committee is concerned not only with programs officially designated as Neurobiology or Excitable Membranes, but with generating new interest in behavior and neurogenesis in established courses, *e.g.*, experimental invertebrate zoology, embryology, and marine ecology.

(2) A year-round resource center for research and advanced study in the genetics, physiology and ecology of marine organisms

It is becoming even more clear that the oceans constitute a life-support system that will become increasingly important to man as population increases and the effects of industrialization and urbanization place greater stresses upon the capacity of the land to support the earth's people. At the same time, pollution of the water is already endangering marine resources. The dangerous levels of mercury recently discovered in fish and in the livers of offshore mammals and of DDT in animals high on the food chain are but two well-publicized examples. Yet although we know that the sea is suffering a massive chemical invasion, we can only guess at the long-term effects, because we know so little about its biology ranging from microbial productivity and degradation to the life cycles and ecological communities in the oceans.

Coping with such long-range problems will require informed leaders, coupled with powerful and sustained scientific efforts, which will in turn mandate the marshalling and interaction of scientists thoroughly trained in the marine biosciences. Such interactions require not only extensive facilities and resources, with access to a variety of marine and estuarine environments, but also of more far-reaching consequence, the development of centers in which the powerful methods of modern biology and chemistry may be brought to bear on the solution of major problems in environmental biology, reproductive physiology and behavior.

Never in the history of American science has a set of problems demanded interdisciplinary research to a greater degree than does the environmental crisis now confronting us. Never have our approaches been so fragmentary, so lacking in depth. It is clearly not enough just to bring ecologists and molecular biologists together. What is required is a common focus, a recurring theme. In the solution of other major problems in the health sciences, that theme has been provided by genetics. We believe that major advances are to be expected from the application of genetics and its related disciplines to the environmental and behavioral sciences. An understanding of the effects of changing the marine environment on any population of organisms will surely require that we know not only their environmental history but their genetic background as well.

Few universities are in a geographic location that would permit them to develop such a program in the marine sciences; furthermore the facilities, resources and staff required by such an enterprise would be prohibitive to all but a few. One practical and effective plan would be to concentrate the national effort in a few national centers for advanced study and research in marine biology to which scientists in training as well as those already experienced but seeking new opportunities for the application of their skills to today's problems would come for circumscribed periods from institutions throughout the nation.

We believe that because of its unique qualities and interdisciplinary tradition, the Marine Biological Laboratory is well equipped to develop such a center. We propose that over the next decade the Laboratory greatly augment and reinforce its capability for serving the nation as a year-round center of advanced study and research in the genetics, physiology and ecology of marine organisms. The specific objectives of this proposal covering the first five years of the program, are threefold:

(1) To plan and construct a Marine Resources Building, embodying environmentally controlled facilities suitable for the development of genetic strains of selected marine species and to initiate long-term studies of the influences of changing environmental conditions on their life histories and behavior. As the program develops, we propose to identify and appoint a nucleus of talented investigators who wish to play a role in developing the field of the physiological genetics of marine organisms.

(2) To initiate a program of Visiting Scholars, designed to bring to the Laboratory a group of investigators whose interests and qualifications bear directly on our long-range goals. This group would include both experienced investigators and younger scientists, who would interact with the first staff scientists to be appointed in the Resource Center, thereby providing a "critical mass" of research talent. We envisage that the fully-developed program will include a substantial nucleus of full-time key investigators in residence and that the Visiting Scholars in Residence would provide a pool of talent from which future staff members may be drawn.

(3) To provide core support to strengthen the Laboratory and permit the coordination of interdisciplinary research and teaching on a year-round basis. The development of the Laboratory's own year-round programs should in turn complement and enhance its efforts to provide inland universities and colleges with access to facilities for education in the marine biosciences.

We believe that the Marine Biological Laboratory constitutes a national resource and that the assurance of its continued development is in the national interest. To meet these objectives, we are seeking major support from the National Science Foundation and other agencies.

In taking these steps, the officers and Trustees have acted in the conviction that the Laboratory must develop year-round and winter activities, while holding fast to the best features of its renowned summer programs. The decision to ultimately commit up to ten per cent of the Laboratory's research facilities to year-round was not an easy one, for on this question the Laboratory has a split personality. We want winter programs, yet we appear to fear them. I wish to make clear my belief that the Laboratory cannot fullfill its responsibilities or long maintain its current strength without a nucleus of excellence in sustained winter research. If winter teaching at the Laboratory is to increase (as I believe it must) then excellence in research the year-round becomes vital; for we will attract the most able winter visitors and students only if there are investigators with whom they can interact. Without a strong research group, we are likely to get second-raters. Bad science drives out good.

Such a nucleus need not be large; quality is what is of importance. I believe that first rate winter programs in one or more fundamental areas of biology will strengthen the summer programs. The choice of areas was made difficult by the pressures being developed for applied science. The most disastrous course of all would have been to compromise our aims by yielding to temptation for "easy money" (if indeed there is *any* easy money). We still have the status to build from strength. We need not adopt indecisive, halfway measures. If we wait the chances for action will only diminish.

If adequate funding can be obtained, we hope to take the first steps in these new directions in 1972. By "adequate funding," however, we mean more than just funds earmarked for the new ventures.

There must be a parallel improvement in our research services, with special attention to the needs of the Supply Department. Our research equipment also badly needs to be upgraded. In 1969 and 1970 our cash deficits amounted to $25,122 and $75,496. These deficits would have been substantially larger had we not deferred the purchase of badly-needed equipment. To the casual observer, especially of the summer scene, we are well-equipped—but much of the equipment in evidence is borrowed or rented, often at only a fraction of the usual cost. Other services need to be upgraded, including electronics, electron microscopy and photography; and the library's resources need to be restored to full-strength.

Scientifically, ways have to be found to bolster our overall strength in the environmental sciences. We have islands of interest—the marine ecology course, the Systematics-Ecology Program—but our efforts lack cohesion. Several recent developments augur well for the future, notably Holger Jannasch's acceptance, during 1970, of the Laboratory's invitation to direct the marine ecology course beginning in 1971, and the close collaboration in research of members of BUMP, SEP and WHOI.

Finally we must innovate without growth of the summer population—a difficult task. We wish to increase the quality of life in Woods Hole, not the quantity.

Winter teaching

The Laboratory plans to initiate vigorous winter courses. In the academic year 1972–1973, we propose to start at least two programs: (1) A semester in Marine Biology (for graduate students) and (2) A January Short Term (one month) in Marine Biology for undergraduates. It is possible that the latter may get underway as early as January, 1972.

Before discussing our ideas about the specific programs, I wish to delineate the thinking behind these decisions.

Over the past several years a number of educational experiments have been initiated at MBL. Of these, the best developed has been the Boston University Marine Program (BUMP). This is a graduate level program in marine biology inaugurated by Boston University. The major component is based at MBL, although the program benefits from courses and research training at the New England Aquarium in Boston and at the Boston campus of the University. During the academic year graduate level courses are offered by BUMP faculty in residence at MBL, where dissertation research is conducted. The Boston University Marine Program is coordinated closely with MBL's research program in Systematics and Ecology (M. R. Carriker, Director) and draws up SEP's facilities.

The staff of BUMP in 1970–71 includes Professor Arthur G. Humes, Director, instructor in marine invertebrate zoology, Assistant Professor Ivan Valiela, instructor in marine ecology, and Assistant Professor William C. Stewart, instructor in environmental physiology. The courses offered are intensive six-week units, accompanied by related seminars and opportunities for research. Qualified graduate students from any college or university may enroll in the courses currently offered, with students from schools other than Boston University receiving credit from their home institutions. Cooperative arrangements are currently being made whereby a limited number of students in BUMP and at the Woods Hole Oceanographic Institution may receive transferable credit for courses either at BUMP or at WHOI.

In 1970 there were nine graduate students in the program, three in their second year at MBL and six newly enrolled. The course in marine invertebrate zoology emphasized the morphology, identification, and habitats of local invertebrates, together with a discussion of phylogeny and systematics. There were 12 field trips (six on board the R/V A. E. VERRILL), 13 lectures, and nine seminars by specialists from MBL, WHOI, the National Marine Fisheries service, and Boston University. In addition, each student carried out a small research project. The course in marine ecology dealt with models in ecology, experiments and design including computer work, population ecology, competition, ecosystems, and community structure and development. The course in environmental physiology is being offered for the first time in the spring of 1971. Students in BUMP have available to them courses at WHOI in biological oceanography, chemical oceanography, and marine geology.

One of the first questions to be raised is whether the MBL should enter into a "working agreement" with Boston University, or with any other university, in elaborating additional winter instructional programs. The Executive and Planning Committees, the Sub-Committee on Winter Educational Activities, and the Instruction Committee have all considered this question. There is virtually unanimous agreement that the BUMP experiment is off to a good start; that in the person of Dr. Humes it has an able and respected leader; and that Dr. Valiela and Dr. Stewart are competent young scientists and teachers. Although the program is a Boston University program, principally geared to the needs of the University's own students, courses are open to qualified students from any university with transfer of credit. Boston University is earnestly supporting BUMP, as evidenced by the 1970–71 budget for the program of approximately $70,000 including salaries. We confidently expect that BUMP will grow in quality and serve an increasingly important role. We hope, in fact, that students enrolling in an MBL semester may have an opportunity of electing a BUMP course or courses. However, we very much want to develop MBL courses with the Laboratory's own special "stamp" on them. We believe that many of the nation's better graduate students should have the opportunity of a full semester of marine biology of the quality of, say, the Laboratory's summer course in physiology.

There has been considerable discussion as to whether the MBL should attempt to develop its teaching program by acting as a focus for the development of a consortium. In fact, the Laboratory has been doing this on a modest scale for some years by providing colleges and universities with laboratory, boat and other facilities for students brought in groups for short periods of intensive study. In 1969–1970 eight colleges and universities including Amherst, Brown, SUNY and Drew were involved in this program. In May 1971 Temple University will offer a month-long course in Marine Invertebrate Zoology at MBL for 24 students. Arrangements are being made for Brooklyn College and the University of Copenhagen to bring students for specified periods in the falls of 1971 and 1972, respectively. In 1970–71, Bridgewater State College is using the MBL's facilities (laboratories, library) on weekends for its course, Intertidal Biology.

In addition, during 1970 discussions were begun with the Bates, Bowdoin and Colby Consortium, looking toward the establishment of a winter research participation program for undergraduates.

Should a broadly representative regional consortium be established, giving more structure and coordination to these programs? A model might be the program at the University of Southern California's Santa Catalina Marine Biological Laboratory, which has working ties with California Institute of Technology, the California State College System, Occidental and Pomona Colleges and the University of California campuses at Irvine, Los Angeles and Riverside. It is possible that in the long run, the development of a consortium may indeed be the most effective mechanism. However, since the MBL is a national, indeed international, center, operating at a high level of quality, we believe that we can ourselves develop several types of instructional programs. To insure administrative control, including the coordination of schedules, the Director, or his designated professional representative, with the advice and aid of the Instruction Committee, should be in residence.

The foregoing decisions stemmed from the report of the subcommittee on Education which is endorsed by the Planning and Executive Committees.

The semester in marine biology at MBL. We propose to initiate this program during the fall term of the academic year 1972–1973. In the first two or three years of the program we expect to offer opportunities to 20–24 beginning graduate students annually. (The group might include a few exceptional undergraduates.) Each student will be enrolled during the term in two intensive courses, at the level of the MBL's traditional summer offerings, including research participation. It has not yet been decided whether the courses will run concurrently or in series, although in order to coordinate them with the BUMP and WHOI programs, it may be necessary to have them run concurrently. The MBL will mount two courses; these together with the BUMP offerings should give each student in the Woods Hole community at least four courses from which to elect a program. One of the MBL courses will be Marine Developmental Biology and the other probably Experimental Invertebrate Zoology, with emphasis on environmental physiology, including endocrine and neural coordination and behavior.

The faculty will be recruited from colleges and universities, both U. S. and European. In the first experimental year, we expect to draw as much as possible from the "MBL community," *i.e.*, Corporation members, former instructors, *etc.*, in order to insure a faculty fully aware of the manner in which we like to function. We hope to be able to appoint key instructors by March 1, 1972, to permit the publication of an announcement of the program by mid-March. Each course will have at least an instructor-in-charge and one other staff member in residence throughout the term, with additional staff members in residence for shorter periods.

The January short term at MBL. We hope to present this program for the first time in January, 1972. We will provide instruction for 20–24 college juniors and seniors. At least one intensive research-oriented course will be offered, Marine Developmental Biology and Reproductive Physiology. Each student will be enrolled in only one course, which will be a full-time effort.

The faculty will be recruited from two sources, from the MBL community and from among the faculties of four-year colleges that have adopted 4–1–4 programs. According to the *Interim Term Digest*, prepared by J. L. Armstrong of Macalester College (October, 1969), at least 150 colleges had adopted 4–1–4 programs.

The involvement of four-year colleges in MBL programs is hardly a new idea. One little-mentioned role of the Laboratory is its function as a place for the carrying out of research by faculty of four-year colleges, and the provision of advanced training, as well as exposure to research environment, to the students of the same institutions.

The Director proposes to take an active part in the first year's Developmental Biology course, along with Professor Edgar Zwilling of Brandeis University, and others.

MBL as a winter meeting and conference center

One desirable feature for a national scientific center is contact with diverse current developments. These the Laboratory has in almost superabundance during the summer.

We have now developed the Laboratory to serve these needs the year-round. It has so functioned in the past in the spring and fall, despite inadequate housing. For example the Society of General Physiologists has long had its annual (fall) meeting at the Laboratory.

The following *advertisement* is intended for those members of the Corporation and other readers who have not visited the Laboratory recently.

With the opening of a new dormitory-dining hall in April, 1971 the Laboratory is almost ideally suited as a meeting and conference center. With 110 rooms (208 beds) and a dining room with 362 places the Laboratory can accommodate meetings of smaller scientific and educational organizations, regional meetings of larger societies, and conferences on special topics, like the Gordon Conferences. Topics such as the relevance and application of biological research to societal problems today, and in the future, would be very appropriate.

There are several possible advantages to users: reasonable fees, attractive environment, isolated enough to keep the group together yet close enough to a major transport center to make access easy, a staff familiar with technical and educational needs, and generally a more friendly reception than a commercial operation might offer. The lecture halls are also designed for their intended use and are not converted ballrooms. In some cases the library would be a unique advantage. For the MBL, conferences should increase the attractiveness and vitality of the Laboratory during the winter months.

The kitchen is open the year round. During the winter the staff of the MBL, the Oceanographic Institution (WHOI) and National Marine Fisheries Service use the dining hall, primarily for lunch. This should not interfere with any meeting since the dining facilities are larger than the number of beds. A coffee shop will also be available. There are three large lecture halls (520, 140 and 75 seats) and at least eight conference rooms seating 10 to 20 persons. WE ARE ANXIOUS TO HAVE THIS BEAUTIFUL NEW BUILDING USED TO FULL CAPACITY THE YEAR-ROUND

Frontiers in research and teaching: an experimental program in the neural sciences

With the support of the National Institute of Neurological Diseases and Stroke, the Laboratory is initiating an experimental program designed to introduce increasing numbers of well-qualified scientists of minority ethnic groups into the neurosciences. In 1971, its first summer, the Frontiers Program will itself be an experiment. The number of Fellows to be appointed will be small (four to eight), but the full range of the Laboratory's instructional and research resources will be available to them.

Alan Steinbach will serve as Coordinator of the program, which is designed to provide Fellows with an opportunity to carry out a personalized program in one or more areas of research and teaching in the biological sciences. Emphasis will be placed on neurobiology, but there will be considerable flexibility. The design of programs will be tailored to the interests and the need of individuals.

The fellowship program is designed primarily for individuals at the doctorate level who, although they have had training in research, desire additional opportunities to obtain research experience and/or additional training. Included is the opportunity to participate in or audit one or more of the summer courses, all of which focus on current research in the particular field. Applicants might be, for example, individuals who because of their commitment to teaching have not had good opportunities to pursue their research interests and wish to keep alive their contact with the frontiers of research. Although applications are welcomed from any individual who believes he would profit from the experience, they are especially encouraged from individuals from minority ethnic groups.

Opportunities for fellows may include programs based on any of the following, or combinations thereof: (1) An independent research program, specified by the applicant; (2) A research program in association with an ongoing research group at the MBL; (3) Association with one of the established summer courses or training programs.

The laboratory: stability with flux

Despite the emphasis in this report on the Laboratory's new directions, it is clear that the advent of a new Director has not resulted in abrupt or dramatic changes, for the officers and Trustees are guided by policies that have evolved through eight decades. To a few, any change, even evolutionary change, is unwelcome. Increasingly, however, all of us engaged in research and teaching have to reappraise our roles and contributions: What have we done? Why have we done it? What should we be doing? The need for new knowledge in the biological sciences has never been greater, but we have too often failed to make that fact clear. We must seize the initiative in interpreting our aspirations to our leaders and the public.

I realize that I have failed to touch upon the contributions of many of the Laboratory's ongoing activities. However future reports will afford an opportunity to examine other departments critically, occasionally to hark back to their beginnings and to trace their development, leading up to an examination of their present-day role. I hope, too, that in future reports I shall be able to treat one of the most neglected (and underrated) achievements of the Laboratory—its contributions in research.

I would add a few final words. A little over a year ago, as I approached my appointment as Director-designate, I learned that Burr Steinbach was not only admired and respected—but loved. How, I wondered, does an "administrator" command not only respect, but affection as well. I have never been accused of being sentimental. In fact, it was once said that my approach to life was, in one respect, not unlike that of George Catlett Marshall. Dean Acheson recalled that the General expected from him complete and even brutal candor; he had no feelings, the General said, "except those which I reserve for Mrs. Marshall."

During a year of working closely with Burr, however, I discovered the source of that affection. It springs initially from him—from the affection and devotion he has for the Laboratory. His good will pervades the institution, and is amplified in each of us. Feedback mechanisms are common in biology—this is another example. It is only fitting that his affection for us be reciprocated.

1. Memorials

MERKEL HENRY JACOBS

By Warner E. Love

The third Director of the Marine Biological Laboratory, Professor M. H. Jacobs, died in Falmouth June 27, 1970, at age 85. From his earliest postdoctoral years until just last year he was continuously associated with the Laboratory, excluding absences caused by World Wars I and II. He became a corporation member in 1911. He was elected Associate Director of this Laboratory 1925–1926, and Director 1926–1938. He was also in charge of the Physiology Course 1921–1929. His directorship began during the boom years of the late 1920's. The Brick Building (Lillie) and the Brick Dormitory had just been completed. Somewhat more than 300 students and investigators were in attendance. At that time all courses of instruction ran simultaneously and severe overcrowding for a short peak period resulted. To alleviate this situation, the system of staggering the courses was begun in 1929 to spread the load more evenly throughout the summer. Then in the early thirties, the Laboratory felt the depression through decreased income, enrollment and subscriptions for space. Attendance, which had risen to 362 in 1931, fell back to a little more than 300, and it was only by 1937 that attendance had recovered and climbed to a new high, 391. The financial problems of the Laboratory were severe and economic health was only barely maintained by the most drastic curtailment of expenditures. Nevertheless, during Dr. Jacobs' directorship the Devils Lane tract was purchased and all outstanding interest-bearing obligations were liquidated. The disposal of the steam vessel Cayadetta, which had been used for collecting and picnicing, must have been an unpopular act of economy.

Professor Jacobs resigned the directorship in 1938 in order to devote himself more completely to his work. He left the MBL essentially intact and undergoing a process of consolidation which hindsight now tells us formed a strong base upon which growth and expansion could occur after World War II.

Merkel Henry Jacobs, his given name was his mother's maiden name, was born of Pennsylvania Dutch stock at Harrisburg, Pennsylvania, December 6, 1885. He earned both his A.B. in 1905 and his Ph.D. (Zoology) in 1908 at the University of Pennsylvania. After one postdoctoral year in Berlin he was invited back to the Zoology Department of the University of Pennsylvania as an Instructor in Protozoology. He was promoted to Assistant Professor in 1913. In 1918–1919 he was a Captain in the Sanitation Corps. In 1921 he moved to the Physiology Department of the Medical School at the University of Pennsylvania as an Assistant Professor, and in 1923 he was made Professor of General Physiology. He lectured to medical students on permeability, renal physiology, acid-base regulation and blood coagulation. He also played a large role in an inter-departmental extra-medical school program of lectures to graduate students on a variety of subjects in General Physiology. All his lectures were utterly clear, scientifically organized and exhaustively prepared. They were models of scholarly excellence. He supervised the doctoral research of approximately a dozen graduate students, many of whom spent some of their summers here at Woods Hole in his laboratory or in courses.

Protozoology was his earliest professional interest. Studies of the effect of CO_2 on protozoa lead him to questions of permeability and by 1921, when he joined the medical faculty, he had become firmly attached to the erythrocyte as his experimental material. Perhaps the most important thread running through all his work was the idea that chemistry, physics, and mathematics should afford explanations for biological phenomena. In collaboration primarily with two former students, Dorothy Stewart and Arthur Parpart, he published a series of about a dozen papers which dealt quantitatively with a number of permeability problems in *Arbacia* eggs and erythrocytes. In 1935 he published a review on Diffusion in Biological Systems in *Ergebnisse der Biologie.* Much to his satisfaction and delight, some thirty years after it appeared, this review was reprinted as a monograph a year or so ago.

The rigor and excellence of his work were recognized and marked by election to the American Philosophical Society and to the National Academy of Science. He served diligently and conscientiously on the editorial boards of five journals and was vice president of the Zoological Society in 1928 and president in 1938. He was a member of a number of scientific societies and a founding member, here at MBL, of the Society of General Physiologists.

Quiet, shy, retiring, and diffident are words to describe only part of the man. He was tenacious of purpose, very hard working, high-principled, and kept his own council. He spoke ill of no one. To those around him, he was above all, gentle.

On a mountaineering expedition on the Selkirk Range of the Canadian Rockies in June 1908, he broke his leg. E. Newton Harvey, in the Festschrift volume # 47 of the *J. Cell. Comp. Physiol.* has described his recollections of participation in the rescue. The leg was set and the party remained in camp till August. During the knitting of the fracture, Professor Jacobs taught himself calculus which stood him in good stead many years later. At one stage of the return journey, transport was provided by lashing him on his stretcher to the cowcatcher of a steam locomotive. The accident left him with a limp which in no way hampered his vigorous pursuit of the joys of walking.

In Philadelphia, even in the dead of winter, on his way in from Media, he detrained one stop early and walked 13 blocks to work, and reversed the procedure to go home.

In 1955 Dr. Jacobs became an Emeritus Professor, having reached the mandatory retirement age of 70. He continued to work both at the University of Pennsylvania and here in the Library at MBL. His 70th birthday was memorialized by volume #47 of the *J. Cell. Comp. Physiol.* In 1961 he received an award for distinguished teaching from the Linback Foundation.

His long life is now finished. He lives on in the minds of those who were privileged to know him. He is perhaps best memorialized by his published work and by the prudence with which he guided the MBL during his directorship through the difficult years of the Great American Depression.

ALFRED HENRY STURTEVANT

By D. E. Lancefield

Alfred Henry Sturtevant was born in Jacksonville, Illinois, on Nobember 21, 1891, and grew up in Alabama. He received the A.B. degree in 1912 and the Ph.D. in 1914 from Columbia. He was immediately given a research appointment from the Carnegie Institution to work with T. H. Morgan, 1915–28. When Morgan went to the California Institute of Technology to establish the Division of Biology in 1928, Sturtevant received a professorial appointment there. Thereafter he held the T. H. Morgan Professorship of Genetics from 1947 to 1962.

Sturtevant first came to the MBL in 1913 and spent most of his summers here since. He served the Laboratory as Trustee for several terms.

In the "Fly Room" at Columbia, Sturtevant was intimately associated with a unique group consisting of Morgan, Bridges, and Muller—to name the most notable. It was an autocatalytic group which accomplished great things in genetic research: no elaboration is needed here.

Sturtevant's dissertation established a major principle of the chromosome theory of heredity, namely the linear order of the genes. He went on from there, and his early work on the comparative genetics of related species of *Drosophila* with its evolutinary significance was important, and remained a lifetime interest. I refer to the crosses between *D. melanogaster* and *simulans*, and to his later pioneer work on the use of polytene chromosomes in phylogeny. His discovery of inversions and the elucidation of their effect on crossing was notable. His analysis of the events associated with "Bar" reversion to normal helped to clear up that puzzling situation.

The diversity of his interests is shown by his published results of investigations on *Oenothera*, irises, man, and horses. His industry is attested to by 79 listings in the MBL Library, which include two monographs and three books.

He was a man with a sound critical judgment and wide acquaintance with the literature. This made him much sought after. He was a visiting professor at three universities here and three in England.

Dr Sturtevant was elected to membership in the National Academy of Sciences in 1930; the American Philosophical Society in 1936. He became President of the Society of Zoologists in 1934, and of the Genetics Society in 1944. Honorary doctorates were conferred on him by Princeton, Pennsylvania, and Yale. For the sake of brevity the list of his honors and awards, culminating in the National Medal of Science in 1968, is left incomplete.

Sturtevant was a fine collector, and his collecting net, as well as his pipe, always went with him. His knowledge of the flora and fauna all over the country made him a first-class naturalist. He married Phoebe Reed in 1922 and one of his special pleasures was his family life. He took great interest in the professional careers of his three children.

He remained a simple and likeable man who was a fine companion whether in genetic discussion or on a camping trip by canoe or car, or on the collecting trips which he so much enjoyed. He ranks as one of the great scientists of our day.

CHARLES PACKARD

By Roberts Rugh

Dr. Charles Packard, the fourth Director of the Marine Biological Laboratory in Woods Hole, died on Monday, March 9th at the Falmouth Hospital. He lived sixteen years beyond the biblical allotment of three score and ten, being 86 years old this year. Of those 86 years 61 were spent entirely or in part in Woods Hole.

Dr. Packard was born in Dorchester, graduated from Syracuse University in 1907, was an instructor in zoology at Williams College for 2 years and then transferred to Columbia where, as an Instructor to such as Bowen, Sturtevant, Gowen and Severinghaus, he worked for his doctorate which he achieved in 1914. The next year he married Marguerite Adams Cogswell, another biology teacher, who survives him. He taught zoology at Williams College until 1918 when he and Mrs. Packard left for a 5 year stay in Peking, China. There he was an assistant professor at the Rockefeller Foundation Union Medical College known as P. U. M. C. For his last year there he acted as chairman of the biology department at Yenching University. He learned to speak Mandarin fluently. His next association was with the Crocker Institute for Cancer Research in New York in 1924, where he rose to the rank of full professor and retired in 1942. During several of the intervening summers he was an instructor on the Embryology Staff in the Woods Hole course. In 1938 he received an honorary doctorate from Syracuse University.

Following Drs. Whitman, Lillie and Jacobs as Director of this laboratory would normally be a difficult assignment. However, Dr. Packard was well prepared as he had been Clerk of the Corporation from 1931–1938 and assistant director for three years while the Trustees searched for a full-time person. Since the Packards had moved to Woods Hole in 1929 they found in Dr. Packard such a person, properly apprenticed, who would be a natural year-round steward of the laboratory affairs. He became Director in 1940 only to retire a second time in 1949. He was an active Trustee from 1949–1954 when he was elected Trustee emeritus.

Dr. Packard's directorship of this laboratory was disorganized by the invasion by the U. S. Navy during the closing years of the second world war. I have heard him say that he survived one war and two hurricanes as Director. The Navy used our Mess Hall facilities for their dining room, as well as some of the lecture halls and domitories so that it was not unusual for the Director to be called on the phone by a girl student or employee asking what she should do about a sailor in her closet Research was drastically curtailed, but the war had to be prosecuted and the sailors trained so that Dr. Packard had the unusual responsibility of trying to balance the demands of the Navy against the earnest endeavors of dedicated scientific investigators limited now both in facilities and time to accomplish what they could not do during their academic years. Following the armistice it fell to Dr. Packard to reclaim from the Navy as much of the Laboratory and its facilities as he could for use of the civilians dedicated to research.

Dr. Packard's scientific interests lay in the area of radiation effects on invertebrate ova such as *Nereis*, *Chaetopterus*, and *Drosophila*. He tried to understand and bridge the gap between the physical stimulation and the biological response; between the effects of Beta, gamma, x- and radium emanations as well as wave length relations in biological reactions. He was one of the pioneers in investigating the biological effects of ionizing radiations, a field that is currently very active. He was on the National Research Foundation Committee for radiation research, a member of the Society of Naturalists and Zoologists, Sigma Xi, and was listed in the American Men of Science.

Like many scientists, Dr. Packard was musically inclined and gifted, playing the flute and piano, singing in the Episcopal Church and the choral club. He was treasurer of the rapidly growing Woods Hole library, served on the town's finance committee and its sewer commission, hoping to help reduce the area pollution.

Dr. Packard gave one the impression of unfailing self-control, of a certain dignity without coldness, of being apart from but not unsympathetic to any problems whether involving personnel or facilities. When confronted with a new problem he would take his pipe from his mouth and say, "Well, well" as he marshalled his thoughts to solve the problem. He thoroughly enjoyed the many Sunday afternoons when he and Mrs. Packard held open-house for any and all members of the Corporation and community. He told one story about a frog which only he could tell, as he was asked to do annually. Dr. Severinghaus, who knew him very well, says he had few equals as a raconteur. He had two hobbies: He kept a daily weather chart presumably hoping against experience of all weather prophets to make something logical out of the weather. He was never able to do this. His second hobby was wood carving and the repair of antique furniture, samples of which are currently in his home.

Dr. Packard in his quiet but dependable way has left his unmeasurable imprint on this institution, and is in part responsible for its present magnificence.

2. The Staff

EMBRYOLOGY

I. Consultant

Everett Anderson, Professor of Biology, University of Massachusetts

II. Instructors

Malcolm S. Steinberg, Professor of Biology, Princeton University, in charge of course
John M. Arnold, Assistant Professor of Cytology, Pacific Biomedical Research Center, University of Hawaii
Max Burger, Associate Professor of Biology, Princeton University
Gary Freeman, Assistant Professor of Biology, University of California at San Diego
Ralph T. Hinegardner, Associate Professor of Biology, University of California at Santa Cruz
Antone Jacobson, Professor of Biology, University of Texas
Hans Laufer, Associate Professor of Zoology, University of Connecticut

III. Lecturers

Raymond Rappaport, Professor of Biology, Union College
Paul B. Weisz, Professor of Biology, Brown University

IV. Laboratory Assistants

Anthony W. Shermoen, Wesleyan University
Robert S. Turner, University of Oregon

V. Lectures

H. Burr Steinbach	Introduction to the Marine Biological Laboratory
M. S. Steinberg	Introduction to the course

Antone Jacobson	Introduction to the teleosts
	Development of teleosts
Everett Anderson	Fine Structure of Eggs (I and II)
Frank J. Longo	Ultrastructural aspects of fertilization
M. S. Steinberg	Morphogenetic phenomena in sponges
	Developmental control processes in coelenterate ontogeny (I and II)
Richard Miller	Pre-fertilization phenomena in hydroids
R. Hinegardner	Echinoderm development: egg to pluteus
	Echinoderms: life cycle and experimental embrology
Charles Helmstetter	Regulation of chromosome replication and cell division in *E. coli.*
M. M. Burger	Cell surface chemistry and the regulation of cell divisions in tissue culture
Raymond Rappaport	Cytokinesis: establishment of the mechanism
	Cytokinesis: nature and operation of the mechanism
John Arnold	Early development in spiralian embryos
	Analysis of molluscan development
	Experimental studies on cephalopod development
R. Weber	Biochemical aspects of tissue involution in the tadpole tail
Charles Emerson	Regulation of DNA-like RNA and the apparent regulation of ribosomal RNA synthesis during development of sea urchin embryos
Antone Jacobson	Experiments on the control of organ determination
Lester Barth	The role of cations in neural induction
Beth Burnside	Neural plate formation and the problem of cell elongation
Malcolm Steinberg	How cells self-assemble into tissues and organs
Richard Sidman	Genetic analysis of morphogenesis in the mammalian brain
Tom Humphreys	Isolation and characterization of factors involved in cell aggregation
Hans Laufer	Embryonic development of crustacea
	Post-embryonic development of crustacea
Irwin Konigsberg	Fusion in cultured embryonic myoblasts
Richard Cloney	Tail absorption in ascidians
Eduardo Scarano	A novel origin of some DNA thymine and its possible role in cell differentiation
Gary Freeman	Development of ascidians
Roger Milkman	Genetics and development of *Botryllus schlosseri*
Gary Freeman	Metamorphosis and asexual reproduction in ascidians
Steven Jay	The relationship of ontogeny to phylogeny
Paul B. Weisz	The significance of larvae
Lionel Jaffe	On the centripetal course of development, the *Fucus* egg and self-electrophoresis
Barry Kiefer	Chemical induction of mitotic abnormalities in sea urchin embryos: cause and consequences
Eric Davidson	Function of repetitive and non-repetitive DNA sequences in oogenesis and early development

VI. Post Course Period

Max Burger, John Arnold and E. Anderson	Biochemical and ultrastructural methods

PHYSIOLOGY

I. Consultants

Albert Szent-Györgyi, Director, The Institute for Muscle Research, Marine Biological Laboratory
W. D. McElroy, National Science Foundation
J. Woodland Hastings, Professor of Biology, Harvard University

II. Instructors

Andrew G. Szent-Györgyi, Professor of Biology, Brandeis University, in charge of course
Sydney Brenner, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England
Roderick K. Clayton, Professor of Biophysics, Cornell University
Hugh E. Huxley, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England
Harvey F. Lodish, Assistant Professor of Biology, Massachusetts Institute of Technology,
Maurice Sussman, Professor of Biology, Brandeis University
David A. Yphantis, Professor of Biology, University of Connecticut

III. Special Lecturers

Harlyn Halvorson, Professor of Bacteriology, University of Wisconsin
K. E. Van Holde, Professor of Physical Chemistry, University of Oregon

IV. Staff Associates

Raymond E. Stephens, Department of Biology, Brandeis University
Annemarie Weber, Department of Biochemistry, St. Louis University
Richard J. Podolsky, Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases
Peter Newell, Department of Biochemistry, Oxford University, Oxford, England
Walter F. Strafford, III, Department of Biophysics, University of Connecticut
Michael Johnson, Department of Biophysics, University of Connecticut
Darrell Fleischman, Charles F. Kettering Research Laboratory
John Finch, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England
David Rekosh, Department of Biology, Massachusetts Institute of Technology

V. Research Assistants

Richard Wayne Linck, Department of Biology, Brandeis University
Ruth Hoffman, Department of Biology, Brandeis University
Jacob Franke, Department of Biology, Brandeis University
Pamela Johnson, Department of Biophysics, University of Connecticut
B. J. Clayton, Department of Genetics, Development and Physiology, Cornell University
Marion Jacobson, Department of Biology, Massachusetts Institute of Technology

VI. Course Assistant

Margaret Ketchum, Cambridge Friends School, Cambridge, Massachusetts

VII. LECTURES

Andrew G. Szent-Györgyi	Aspects of the chemistry of muscle contraction Paramyosin assembly and the filaments of molluscan "catch" muscles
Annemarie Weber	Control of contraction and relaxation
R. K. Clayton	Photosynthesis
	Photosynthetic reaction centers
Darrell E. Fleischman	Photosynthetic membranes: optical and electrochemical properties
David A. Yphantis	Physical approaches in biochemistry (I, II and III)
K. E. Van Holde	Ligand binding by giant molecules
R. E. Stephens	Function, biochemistry and philosophy of microtubules
Richard J. Podolsky	Control of muscle contraction
Irvin Isenberg	DNA-polylysine interaction
J. Woodland Hastings	Luciferase of temperature sensitive mutants of luminous bacteria
Emanuel Margoliash	The tertiary structure of cytochrome C —antibodies as probes of surface functions
	Evolutionary implications of primary and tertiary structure variations in cyctochrome C
Laszlo Lorand	Transpeptidase-controlled assembly of fibrin
Harvey F. Lodish	Regulation of the transformation of the genes of bacterial phage RNA
	The mechanism of initiation of mammalian protein synthesis
	The life cycle of the simplest virus—the RNA-bacterial phage
Rachmiel Levine	Hormones and membranes
Maurice Sussman	DNA in eucaryotes
	RNA in eucaryotes
	The thoughts of Chairman Mao on cellular slime molds
H. G. Callan	Organization of genetic units in chromosomes
Harlyn Halvorson	The Norwegian question: temperature dependent sex in saccharomyces
B. P. Schoenborn	Neutron diffraction for biological structures
Zack Hall	Acetylcholinesterase at the neuromuscular junction
Howard K. Schachman	Proteins and subunits
H. E. Huxley	X-ray diffraction studies on muscle—general review and future prospects
	Current problems in the structure and function of muscle and some other motile systems
J. T. Finch	Analysis of electron micrographs of periodic structures by optical diffraction and computation
Moshe Shilo	Toxigenic phytoflagellates
Albert Szent-Györgyi	Water, matter and matrix
Bessel Kok	Cooperation of positive charges in photosynthetic oxygen evolution
Lawrence B. Cohen	Changes in axon structure during activity
Richard Cone	Rhodospin and visual excitation
Akimichi Kaneko	Vertebrate retinal neural connections
Margit K. Nass	Mitochondrial DNA
David Baltimore	RNA-tumor virus DNA-polymerase

George Wald	Molecular basis of human vision Ontogeny and phylogeny at the molecular level
Sydney Brenner	Control mechanisms I, II, and III
William Bauer	The chemistry of closed circular DNA
Seymour S. Cohen	Polyamines in the structure and function of nucleic acids
Fotis Kafatos	Developmental studies on insects
Peter von Hippel	The dynamic aspects of DNA structure as studied by hydrogen exchange and chemical probes
Lewis Greene	Pharmacologically active peptides from *Bothrops jararca* inhibitors of the metabolism of bradykinin and angiotensin
David Shepro	The microvascular system: contractile protein activity in hemostasis and in nurturing endothelium
Daniel Morse	Dynamics of synthesis, translation and degradation of tryptophan operon messenger RNA in *E. coli*

VIII. Special Seminars

David Jaffe	Myoblast development by cloned cells

EXPERIMENTAL MARINE BOTANY

I. Consultants

Sterling B. Hendricks, U. S. Department of Agriculture
Bessell Kok, Research Institute for Advanced Studies

II. Instructors

Harold W. Siegelman, Plant Biochemist, Brookhaven National Laboratory, in charge of course
Robert R. L. Guillard, Associate Scientist, Woods Hole Oceanographic Institution
F. T. Haxo, Professor of Marine Biology, University of California at San Diego
Frank A. Loewus, Professor of Biology, State University of New York at Buffalo
John M. Olson, Biophysicist, Brookhaven National Laboratory
Robert T. Wilce, Associate Professor of Botany, University of Massachusetts

III. Special Lecturers

Martin Gibbs, Professor of Biology, Brandeis University
Sarah Gibbs, Associate Professor of Botany, McGill University
Carl A. Price, Professor of Botany, Rutgers University
Jerome Schiff, Professor of Biology, Brandeis University
Ruth Sager, Professor of Biology, Hunter College
Myron Ledbetter, Electron Microscopist, Brookhaven National Laboratory
Philip Thornber, Plant Biochemist, Brookhaven National Laboratory
Edward Carpenter, Assistant Scientist, W. H. O. I.
David Wall, Associate Scientist, W. H. O. I.
William Dunstan, Assistant Scientist, W. H. O. I.
W. Yaphe, Chairman of Department of Microbiology and Immunology, McGill University

IV. Assistants

J. P. Thornber, Brookhaven National Laboratory
G. J. Wagner, State University of New York at Buffalo

V. Lectures

H. W. Siegelman	Phytochrome—the chromoprotein regulating plant growth
	Algal bioproteins and bile pigments
C. A. Price	Zonal centrifugation
R. R. L. Guillard	Influence of environmental factors on phytoplankton growth I and II
E. J. Carpenter	Influence of environmental factors on phytoplankton growth III. Nutrient concentrations
D. Wall	Fossil dinoflagellate life histories
W. M. Dunstan	Influence of environmental factors on phytoplankton growth IV. Light adaptation in marine phytoplankton
Frank A. Loewus	Cyclitols: a new dimension in carbohydrate metabolism
	Pistil secretion product: its role in pollen tube development
	Ascorbic acid metabolism: a re-examination
Jerome Schiff	Sulfate metabolism in algae
Martin Gibbs	Carbon metabolism in photosynthesis
John M. Olson	Light absorption, energy transfer and the photosynthetic unit
	Electron transport in bacteria and O_2-producing organisms
	Evolution of photosynthesis in prokaryotes
J. P. Thornber	Components of photosynthetic membranes
F. T. Haxo	Pigments, light absorption and photosynthesis in marine algae I and II
	Photosynthesis in symbiotic algae and chloroplasts
Ruth Sager	Non-Mendelian inheritance in *Chlamydomonas*
Jerome Schiff	Developmental interactions among cellular compartments in *Euglena*
Robert T. Wilce	Attached algae: major group characteristics I (Morphology and development)
	Attached algae: group characteristics II
	Attached algae: littoral and sublittoral ecology
M. Ledbetter	Plant microtubules
Bessel Kok	Cooperation of positive charges in photosynthetic oxygen evolution (Sponsored jointly with the Physiology Course)
C. A. Price	Theory of density gradient centrifugation: evolution of zonal rotors
	Instrumentation: Current types of rotors, gradient generators, monitoring devices
	Isopycnic and rate-zonal separations: gradient shapes for the optimization of resolution and capacity
	Continuous-flow centrifugation, S-p separations, reorienting gradient centrifugation, choice of gradient materials
	Specific separations

EXPERIMENTAL INVERTEBRATE ZOOLOGY

I. Consultants

Frank A. Brown, Jr., Morrison Professor of Zoology, Northwestern University
C. Ladd Prosser, Professor of Physiology, University of Illinois
Clark P. Read, Professor of Biology, Rice University

Alfred C. Redfield, Woods Hole Oceanographic Institution
W. D. Russell-Hunter, Professor of Zoology, Syracuse University

II. Instructors

James F. Case, Professor of Biology, University of California, Santa Barbara, in charge of course
Alan Gelperin, Assistant Professor of Biology, Princeton University
David C. Grant, Assistant Professor of Biology, Davidson College
Jonathan P. Green, Assistant Professor, Brown University
Michael J. Greenberg, Associate Professor, Florida State University
Joseph B. Jennings, Department of Zoology, University of Leeds
Charlotte P. Mangum, Associate Professor of Biology, College of William and Mary
James G. Morin, Assistant Professor of Zoology, University of California, Los Angeles

III. Special Lecturers

Melbourne R. Carriker, Director, Systematics-Ecology Program, Marine Biological Laboratory
Preston Cloud, Department of Geology, University of California
T. H. Goldsmith, Associate Professor of Biology, Yale University
G. F. Gwilliam, Professor of Biology, Reed College
Clark P. Read, Professor of Biology, Rice University
Thomas J. M. Schopf, Assistant Professor of Geophysical Sciences, University of Chicago
Dorothy M. Skinner, Biology Division, Oak Ridge National Laboratory

IV. Assistants

Eve C. Haberfield, University of Rhode Island, Kingston
Roger C. Halverson, University of California, Santa Barbara
George A. Kahler, III, Rice University

V. Lectures

H. Burr Steinbach	Introduction to the Marine Biological Laboratory
J. F. Case	Introduction to the course
	Porifera
D. C. Grant	Introduction to field trip protocol
J. Morin	Coelenterates, I and II
G. F. Gwilliam	Acoelomates
	Pseudocoelomates
C. P. Mangum	Annelids
M. J. Greenberg	Molluscs, I and II
D. C. Grant	Cape Cod environment
Thomas J. M. Schopf	Biology of moss animals
Jonathan P. Green	Arthropoda, I and II
Melbourne Carriker	Recent studies on boring mechanisms in muricids
Alan Gelperin	Echinoderms
J. F. Case	Protochordates
C. P. Mangum	Respiration, I: exchange, II: transport
Jonathan P. Green	Neuroendocrinology: introduction
T. H. Goldsmith	Arthropod vision

Jonathan P. Green	Life in a box: crustacean molting physiology
	Arthropod endocrinology (concluded)
	Osmo- and ion regulation
M. J. Greenberg	Patterns of circulation among the invertebrates
	Hearts and visceral muscle: the way to a clam's heart is through its rectum
	Some aspects of comparative muscle physiology
D. C. Grant	Community structure and diversity
Clark P. Read	Nutritional mechanisms in animal parasites
Dorothy M. Skinner	Satellite DNA's in crustacea
J. B. Jennings	Alimentary systems
	Nutritional physiology of acoelomates I and II
J. F. Case	Regulatory neural mechanisms
J. Morin	Primitive nervous systems
	Colonial organization
	Coelenterate bioluminescence
J. F. Case	Firefly bioluminescence
	Invertebrate chemoreception
Alan Gelperin	Strategies of behavioral physiology
	Neural regulation of feeding
	Executive neurons
Preston Cloud	Primitive earth

MARINE ECOLOGY

I. Consultants

Melbourne R. Carriker, Director, Systematics-Ecology Program, Marine Biological Laboratory

Howard L. Sanders, Woods Hole Oceanographic Institution

John H. Ryther, Woods Hole Oceanographic Institution

II. Instructors

Lawrence B. Slobodkin, Professor of Biology, State University of New York at Stony Brook, in charge of course

Lev Fishelson, Senior Lecturer, Department of Zoology, University of Tel Aviv, Israel

Ralph Mitchell, Associate Professor, Laboratory of Applied Microbiology, Harvard University

Sumner Richman, Professor of Biology, Lawrence University

W. Rowland Taylor, Associate Professor of Oceanography, Department of Earth and Planetary Sciences and the Chesapeake Bay Institute, The Johns Hopkins University

Edward O. Wilson, Professor of Zoology, Harvard University

III. Special Lecturers

J. Frederick Grassle, Woods Hole Oceanographic Institution

Holgar Jannasch, Woods Hole Oceanographic Institution

John Kanwisher, Woods Hole Oceanographic Institution

Richard Koehn, State University of New York, Stony Brook

K. C. Marshall, University of Tasmania, Tasmania

Howard L. Sanders, Woods Hole Oceanographic Institution
Rudolph Scheltema, Woods Hole Oceanographic Institution
John Teal, Woods Hole Oceanographic Institution
W. Yaphe, McGill University, Montreal

IV. Laboratory Assistants

Herman F. Bosch, Department of Earth and Planetary Sciences and the Chesapeake Bay Institute, The Johns Hopkins University
Wayne H. Bell, Department of Biology, Middlebury College

V. Lectures

W. Rowland Taylor	Chemistry of seawater
	Light penetration in seawater
Lawrence B. Slobodkin	Survey of ecological fields
	Elements of population dynamics
W. Rowland Taylor	Plankton ecology: phytoplankton I and II
	Plankton ecology: primary productivity
	Plankton ecology: productivity methods
Sumner Richman	Zooplankton feeding behavior I and II
Thomas Lawson	Copepod biology
Lawrence B. Slobodkin	How did predators get so clever?
Sumner Richman	Bomb calorimetry
	Food chain dynamics
Herman F. Bosch	The marine Cladocera
Lev Fishelson	Coral reef metabolism and development
	Coral reef growth and organization
	Coral reef destruction
	Symbiosis on coral reefs as a factor regulating species numbers
	Coral reef crinoids: ecology and associated fauna
	Sex reversion and reproductive behavior of the coral fish *Anthias squamippinis*
Ralph Mitchell	What is applied microbial ecology?
Holgar Jannasch	Problems in microbial ecology
Ralph Mitchell	The role of microorganisms in the fouling of surfaces
K. C. Marshall	Interfacial phenomena in microbial ecology
Ralph Mitchell	Reversal of imbalances in microbial systems
	Biological control of undesirable organisms
	The role of polysaccharides in microbial aggregation
Richard Koehn	Biochemical polymorphism in natural fish populations
W. Yaphe	Agar: a polysaccharide of interest to the phycologist, mycologist and biochemist
Lawrence B. Slobodkin	The strategy of evolution: I and II
	Environmental design and decision making
	The population problem
Edward O. Wilson, I. Rubinoff and Howard L. Sanders	Ecological effects of a sea-level canal in Central America
Ralph Mitchell	The scientific approach to water pollution control
Joseph Loya	Coral diversity in the Gulf of Elath

Edward O. Wilson	Colonization and the species equilibrium The analysis of adaptive radiation Chemical communication among organisms Competitive and aggressive behavior The future of sociobiology
J. Frederick Grassle	Species diversity, genetic variation and environmental uncertainty
John Kanwisher	Comparative physiology
Rudolph Scheltema	Dispersal of larvae as a means of genetic exchange between widely separated populations of benthic invertebrate species
John Teal	Effects of hydrostatic pressure on marine organisms
Howard L. Sanders	Marine benthic diversity

NEUROBIOLOGY

I. Instructors

Michael V. L. Bennett, Professor of Anatomy, Albert Einstein College of Medicine, co-director of course

John E. Dowling, Associate Professor of Ophthalmology and Biophysics, Johns Hopkins University School of Medicine, co-director of course

Felix Strumwasser, Professor of Biology, California Institute of Technology

Victor Whittaker, Sir W. Dunn Reader in Biochemistry, Cambridge University

II. Special Lecturers

George Pappas, Professor of Anatomy, Albert Einstein College of Medicine

David L. Wilson, Research Fellow, California Institute of Technology

Jon W. Jacklet, State University of New York, Albany

Bertram Peretz, University of Kentucky

Itzhak Parnas, Visiting Professor, Columbia University

Edith Heilbronn, Associate Professor, University of Uppsala

S. R. Shaw, Visiting Scientific Fellow, National Institutes of Health

Nigel W. Daw, Washington University

III. Lectures

M. V. L. Bennett	The central dogma: I and II
George Pappas	Fine structure of synapses
Felix Strumwasser	The neurocellular basis of behavior in *Aplysia:* I and II
David L. Wilson	Molecular weight distribution of proteins synthesized in single, identified neurons of *Aplysia*
Jon W. Jacklet	The eye of *Aplysia:* light responses and circadian activity
Bertram Peretz	Neural correlates of centrally and peripherally initiated behavior in the gill of *Aplysia*
Itzhak Parnas	Peripheral integration at the level of neuromuscular junctions in arthropods
Victor Whittaker	Biochemical techniques in the study of synaptic function: I, II, and III
Edith Heilbronn	The use of drugs for investigating the mechanism of cholinergic transmission: I and II

M. V. L. Bennett	Functional aspects of electrotonic transmission Properties of receptor synapses Interpretation of intracellularly recorded potentials: spikes Methodology continued: postsynaptic potentials
J. E. Dowling	Vision I: anatomy, chemistry, and physiology review Vision II: receptor potentials
S. R. Shaw	Invertebrate visual systems
J. E. Dowling	Vision III: visual processing
N. W. Daw	Vertebrate color vision
J. E. Dowling	Vision IV: visual deprivation

SYSTEMATICS–ECOLOGY PROGRAM

The Staff

Director: Melbourne R. Carriker

Acting Resident Systematists (Zoology): Paul L. Illg, Arthur G. Humes

Acting Resident Systematist (Botany): Robert T. Wilce

Resident Ecologists: David K. Young, Ivan Valiela

Assistant Ecologist: Katharine D. Hobson

Postdoctoral Fellows and Research Associates: James Fiore, Raymond P. Markel, Lawrence R. McCloskey, Leland W. Pollock, Norman R. Sinclair, William J. Woelkerling

Graduate Research Trainees: William R. Cobb, Joan R. Conway, Mary Ann Gilbert, William H. Gilbert, Walter Hatch, Charles Krebs, Allan D. Michael, Roy M. Yarnell

Visiting Investigators: Edward Bousfield, Louise Bush, Howard H. Chauncey, Edward DeLamater, William D. Hummon, M. Patricia Morse, Joel S. O'Connor, Philip Person, Harold H. Plough, Donald C. Rhoads, William C. Summers, Wesley N. Tiffney, Ruth D. Turner, David K. Young

Consultants: William Randolph Taylor, Ruth D. Turner, Robert T. Wilce

Curator: Johanna M. Reinhart

Assistant Curator (Gray Museum Herbarium): Joan R. Conway

Technical Field Assistant: Peter J. Oldham

Field Assistant: Francis Doohan

Scientific Illustrators: Ruth von Arx, Susan P. Heller

Captains, R/V A. E. Verrill: James P. Ostergard, Peter Graham

Mates, R/V A. E. Verrill: Peter Graham, Francis Doohan

Administrative Assistant: Constance A. Brackett

Program Secretary: Eva S. Montiero

Research Assistants: Susan Anderson, Barry Bluestein, Anne C. Collins, Theodore J. Grant, David J. Hartzband, Richard A. McGrath, Stephen McGrath, John J. McMahon, Carol Q. Schwamb, Anne Smarsh, Martha Speirs, Pamela Tannebring, Laura Tosi, Richard J. Traverse, Andrea Turner, Dirk Vanzandt

Visitors: Greg Mordas, Michael Sweeney, Langley Wood

SEP Seminars (Winter Included)

Patricia L. Dudley	Some aspects of the biology of parasitic and commensal crustacea
H. Burr Steinbach	Woods Hole, a systematic and ecological commentary on a scientific community

John Sutherland	Dynamics of high and low populations of the limpet *Acmea scabra*
R. Stuart Mackay	Decompression sickness: ultrasonic detection of bubbles and fluid breathing
Charles L. Remington	Suture-zones: sites of natural experiments of rapid evolution
James Marsh	Primary productivity of reef-building calcareous red algae
Paul I. Illg	*Dendrogaster*—a cirripede parasite of starfish
John W. Evans	The ecology of the rock boring clam *Penitella penita*
David Pratt	Olfactory analogs of protective coloration ?
David C. Culver	Niche separation and species packing of cave crustaceans
Leonard Ortolano	Marine resources planning in a world of perfect information
Frank Smith	Environmental quality: biological imperatives and economic choices
Raymond P. Markel	Some physiological responses to temperature acclimation in the limpet, *Acmaea limatula*
John Culliney	Larval development and ecology of *Teredo navalis* and *Bankia gouldi*
J. F. Grassle	Saving the Barrier Reef—a case history of conservation
Amelie Scheltema	*Chaetoderma canadense:* the solenogaster of Cape Cod Bay
Malcolm C. Mercer	The biology and fishery of the ommastrephid squid *Ilex illecebrosus*, in the Northwest Atlantic
Authur Mathieson	Ecological studies on marine algae in Great Bay Estuary System, New Hampshire
William H. Gilbert	The relationship between ecological niche and dispersion pattern in populations of bivalve mollusks
Jeffrey Levinton	Stability and trophic structure in deposit-feeding communities
Donald B. Horton	TRIGOM, a new educational consortium: program and prognosis
Peter Roy	Tube dwelling behavior of the sabellarid polychaete *Phragmatotoma californica* Fuwkes
Sylvia Earle	Ecology and distribution of the brown algae of the Gulf of Mexico
Robert Zottoli	The species area curve as applied to the choice of adequate sample size in the rocky intertidal zone
Mary M. Allen	Growth and cell division in blue-green algae
Charles Lambert	Genetic transcription during tunicate development and metamorphosis
Thomas Sweeney	A method of eliminating oil slicks on water by combustion
Craig Edwards	Ecology of *Polinices duplicatus* Say
I. Mackenzie Lamb	Diving for marine algae in antarctica
Richard Fralick	*Codium* in New England
Susan M. Smith	Behavioral adaptations for predation in the loggerhead shrike, *Lanius ludovicianus*
Lawrence R. McCloskey	*Acanthaster*: ecocrisis or pseudoproblem ?
Edward L. Bousfield	The Hudson 70 Expedition in the Cape Horn Region
Galen Jones	Effect of trace elements on marine bacteria
James F. Clark	An experimental underwater habitat
Phillip G. Coates	Some aspects of the winter flounder tagging and research program in Massachusetts
Roger Szal	A "new" sense organ in certain primitive gastropods

Wilbur L. Bullock	Hooks, spines and cement glands: the morphological approach to acanthocephalan systematics
Henry Moeller	Life history and ecology of *Codium fragile* in Eastern Long Island, New York, waters
Richard Tatlock	Aircraft remote sensing in New England (current velocity surveys, infrared thermal scanning, aerial water sampling, aerial XBT development)
Ken Howard	Oogenesis in an Icelandic water mold, *Saprolegnia terrestris* Cookson
John D. Palmer	The ups and downs in the daily life of *Euglena*, a biological rhythm study
William Randolph Taylor	The shallow-water marine algal vegetation of Bermuda and the West Indies
Paul Fell	Some aspects of the growth, reproduction and hibernation of several marine sponges
Darwin Davidson	The physiological ecology of some freshwater and marine ascomycetes

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Slaughter, Margaret Ann, Yale University
Smucker, Luellen A., University of Delaware
Smyth, Ward Alan, Central Connecticut State College
Snyder, David Andrew, Brown University
Sosa, Jorge Sanchez, Boston City Hospital
Stafford, Walter F., III, University of Connecticut
Stephenson, John E., Tulane University
Stillings, Wayne, Oberlin College
Stocks, Adelaine, Columbia University
Stuart, Claude Leroy, III, Princeton University
Suddith, Robert L., Indiana University
Szamier, R. Bruce, Albert Einstein College of Medicine
Szonyi, Estzer I., Harvard Medical School
Terebey, Nicholas, State University of New York, Upstate Medical Center
Tobias, Thomas, University of Pennsylvania
Tocci, Salvatore, Brooklyn College
Toomey, Barbara, Goucher College
Townsend, Kay, University of Minnesota
Tucker, Gail Susan, University of Kansas
Turner, Robert Scott, University of Oregon
Turpen, James B., Tulane University
Twomey, Stanley Lawrence, University of Kansas
Wagner, George J., State University of New York at Buffalo
Waung, Hsi Fong, State University of New York at Stonybrook
Waxman, Stephen G., Albert Einstein College of Medicine
Wexler, Andrew, Dartmouth College
Wolley, Robert C., Tulane University
Young, Janice E., Northwestern University
Yulo, Theresa, University of Rochester Medical Center
Zakevicius, Jane M., New York University Medical Center
Zipser, Birgit, Albert Einstein College of Medicine

Library Readers, 1970

Allen, Garland E., Assistant Professor of Biology, Washington University
Anderson, Rupert S., Independent Library Reader, Marine Biological Laboratory
Augenfeld, John M., Independent Library Reader, Marine Biological Laboratory
Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Bendet, Irwin, Professor of Biophysics, University of Pittsburgh
Berne, Robert M., Professor and Chairman of the Department of Physiology, University of Virginia, School of Medicine
Birnbaum, Allan, Professor, New York University, Courant Institute of Mathematical Sciences
Boettiger, Edward G., Professor of Physiology, University of Connecticut
Bridgeman, Josephine, Professor of Biology, Agnes Scott College
Buck, John, Chief, Laboratory of Physical Biology, National Institutes of Health
Burnside, Mary Beth, Postdoctoral Fellow, Harvard University
Cable, Raymond M., Professor of Biology, Purdue University
Carlson, Francis D., Professor of Biophysics, Johns Hopkins University
Chase, Aurin M., Professor of Biology Emeritus, Princeton University
Clark, Arnold M., Professor of Biological Sciences, University of Delaware
Davis, Bernard D., Professor of Bacterial Physiology, Harvard Medical School
Eder, Howard A., Professor of Medicine, Albert Einstein College of Medicine
Elkins, William L., Assistant Professor, University of Pennsylvania
Gabriel, Mordecai L., Professor and Chairman of Biology, Brooklyn College
Gelfant, Seymour, Professor of Zoology, Syracuse University

German, James, Associate Professor, Department of Anatomy and Pediatrics, Cornell University Medical Center

Ginsberg, Harold S., Professor and Chairman, Department of Microbiology, University of Pennsylvania

Gitlin, David, Professor of Pediatrics, University of Pittsburgh, School of Medicine

Green, James W., Professor of Physiology, Rutgers University

Haugaard, Niels, Professor of Pharmacology, University of Pennsylvania

Hill, Robert B., Associate Professor, University of Rhode Island

Isselbacher, Kurt J., Professor of Medicine, Chief, Gastrointestinal Unit, Massachusetts General Hospital

Kaltenbach, Jane C., Associate Professor, Mount Holyoke College

Kempton, Rudolf T., Professor Emeritus of Biology, Vassar College

Krassner, Stuart M., Associate Professor, University of California, Irvine

Laki, Koloman, Chief, Laboratory of Biophysical Chemistry, National Institute of Arthritis and Metabolic Diseases—LBC

Levine, Rachmiel, Chairman, Department of Medicine, New York University Medical College

Lineaweaver, Thomas H., Independent Library Reader, Marine Biological Laboratory

Luria, S. E., Professor of Biology, Massachusetts Institute of Technology

Mahler, Henry R., Research Professor, Indiana University

Marks, Paul A., Professor and Chairman, Department of Human Genetics and Development, Columbia University, College of Physicians and Surgeons

Marshak, Alfred, Tulane University Medical School

Marsland, Douglas, Research Professor Emeritus, New York University

Mautner, Henry G., Professor and Chairman, Department of Biochemistry and Pharmacology, Tufts University, School of Medicine

Mizell, Merle, Associate Professor Biology, Tulane University

Morrell, Frank, New York Medical College

Nasitir, Maimon, Professor and Chairman, Department of Biology, University of Toledo

Palmer, John D., Chairman, Department of Biology, New York University

Porter, Keith R., Professor of Biology, Harvard University

Rosenberg, Evelyn K., Associate Professor of Biology, Jersey City State College

Rosenkranz, Herbert S., Professor of Microbiology, Columbia University, College of Physicians and Surgeons

Rosine, W. N., Professor of Biology, Augustana College

Roth, Jay S., Professor of Biochemistry, University of Connecticut

Roth, Owen H., Head of Biology Department, St. Vincent College

Rowland, Lewis P., Professor and Chairman Department of Neurology, University of Pennsylvania

Rubinow, Sol I., Professor of Biomathematics, Cornell University Medical College

Sager, Ruth, Professor, Hunter College

Schlee, Susan, Independent Library Reader, Marine Biological Laboratory

Schlesinger, R. Walter, Professor and Chairman, Department of Microbiology, Rutgers University

Scott, Allan, Professor and Chairman, Department of Biology, Colby College

Smelser, George K., Professor of Anatomy, Columbia University, College of Physicians and Surgeons

Sonnenblick, B. P., Professor of Zoology, Rutgers University

Spector, Abraham, Associate Professor of Ophthalmology, Columbia University, College of Physicians and Surgeons

Sperelakis, Nick, Professor Physiology, University of Virginia

Stetten, DeWitt, Jr., Dean and Professor, Experimental Medicine, Rutgers Medical School

Stetten, Margorie R., Research Professor, Experimental Medicine, Rutgers Medical School

Stiller, Ronald A., Graduate Student, Boston University

Strickberger, Monroe W., Associate Professor of Biology, University of Missouri

Thomas, Lewis, Professor and Chairman, Department of Pathology, Yale School of Medicine

Vacca, Linda L., Graduate Student, Tulane University

Wainio, Walter, Professor of Biochemistry, Rutgers University

Weiss, Leon, Professor of Anatomy, Johns Hopkins University

Wheeler, George E., Associate Professor of Biology, Brooklyn College

Wichterman, Ralph, Professor of Biology, Temple University
Wilson, Thomas Hastings, Professor and Chairman, Department of Physiology, Harvard Medical School
Wittenberg, Jonathan B., Professor of Physiology, Albert Einstein College of Medicine
Yntema, Chester, Professor of Anatomy, State University of New York—Syracuse
Zeidenberg, Phillip, Institute in Psychiatry, Columbia, College of Physicians and Surgeons
Zipser, David, Principal Staff Investigator, Cold Spring Harbor Laboratory for Quantitative Biology

Students, 1970

All students listed completed the formal course program, June 16–July 26. Asterisk indicates students completing post-course research program, July 27–August 30.

ECOLOGY

Bari, Gina, Massachusetts Institute of Technology
*Becker, Peter F., Lawrence University
*Davis, B. Jeanne, Scripps Institution of Oceanography
Dunn, Rosalie A., National Biomedical Research Foundation
Eaton, David J., Oberlin College
Flannery, Maureen A., Mount Holyoke College
Grant, Michael G., Texas Technical University
Greenspan, Beverly N., The Rockefeller University
Hardoby, William J., Syracuse University
Kastendiek, Jon E., University of California, Los Angeles
Koral, Stephen M., Harvard University
Manos, Peter J., Harvard University
*Moskol, Ann E., Harvard University
Picardi, Anthony C., Massachusetts Institute of Technology
*Pomerantz, Mark, Reed College
Rosner, Judah L., National Institutes of Health
*Rubenstein, Elaine C., State University of New York at Buffalo
Shaffer, Ellen J., University of Minnesota
Silbergeld, Ellen K., The Johns Hopkins University
Snyder, Alice J., Bryn Mawr College
*Spiller, Judith A., State University of New York at Stony Brook
*Walker, Mary Clare, New York University Medical School
Zoltowski, Carol, Seton Hill College

EMBRYOLOGY

*Conner, Brenda Jean, Emory University
*Courtois, Yves, Massachusetts General Hospital
Darst, Russell P., III, University of North Carolina
*Dasch, Gregory Alan, Oberlin College
*Ducibella, Thomas, Princeton University
*Fritzler, Marvin J., University of Calgary
Fry, Anne E., Ohio Wesleyan University
*Hagedorn, Henry H., University of California, Davis
Johnston, Michael A., Yale University
*Kuhns, William J., New York University School of Medicine
Le Count, Thomas Samuel, University of California, Davis
*Leith, Ardean, University of Rochester
Lo, Timothy, Illinois Institute of Technology
Loftfield, Robert B., University of New Mexico, School of Medicine
Macarak, Edward J., University of Pennsylvania
*Miyamoto, David M., University of California, San Diego
Morek, Sr. Dolores M., University of Notre Dame
Nickerson, Kenneth W., University of Wisconsin

NEIDERMAN, RICHARD, University of California, Davis
POCCIA, DOMINIC L., Harvard University
PUKKILA, PATRICIA J., University of Wisconsin
ROSSETTI, PETER L., State University of New York, Upstate Medical Center
SCHWARTZ, MARCIA F., The Johns Hopkins University
TURNER, DAVID C., National Heart and Lung Institute, National Institutes of Health
VAN DENBOS, GARY, Graduate School of Biomedical Sciences, Oak Ridge National Laboratory
WEBB, GLENDA C., The Johns Hopkins University
WEBER, LEE A., University of Connecticut
YINGLING, WENDY B., Rice University

EXPERIMENTAL BOTANY

*ALBERTE, RANDALL S., Duke University
*BERNTSEN, BARBARA, University of Oregon, Eugene
BOWLER, PETER A., Bard College
CRAWFORD, GREGORY, University of Miami
DARWIN, STEVEN P., Drew University
*DUNAWAY, CHARLES L., University of Alabama
*LAFFERTY, MARY ANN, University of Virginia
MARKOWITZ, MELVIN M., University of Illinois
*MARTIN, MARY A., University of Massachusetts, Amherst
NIKLAS, KARL J., University of Illinois
*PERRY, MARY JANE, Scripps Institution of Oceanography
RICHARDSON, CHILTON A., Chatham College
RUBIN, PAULA S., University of Texas
STAKER, ROBERT D., University of Arizona
*STEINBACK, KATHERINE E., University of California, Berkeley
TEGNER, MIA J., Scripps Institution of Oceanography
*WEISTROP, JESSIE S., University of Massachusetts
*WETHERBEE, RICHARD, University of Michigan
WILUSZ, CAROL A., University of Massachusetts

PHYSIOLOGY

*BAKER, WILLIAM BRADFORD, University of Illinois, Urbana
*BONNER, ROBERT FRANCIS, Johns Hopkins University
*CARPENTER, DONALD ELLIS, Oregon State University
*CHENEY, CAROL, Oberlin College
DRAPER, MICHAEL WILLIAM, The Rockefeller University
*GOULD, JOHN HOWARD, University of Massachusetts, Amherst
*HEREFORD, LYNNA MADSEN, Yale University Medical School
*HOFFMAN, PETER ROBERT, College of Physicians and Surgeons, Columbia University
*HUANG, DONNA D. C., University of Pennsylvania Medical School
*JOHNSON, PAUL ANDREW, Yale University
*KLEIN, NATALIE C., New York University
*KOPPENHEFFER, THOMAS L., Boston University
*LEVIN, SUSANNE, Brown University
*LINDNER, ROBERT, Northwestern University
*LINDSTROM, DONA MEI, University of California, San Diego
NORDEN, ANTHONY, University College, London, England
*POTTER, JAMES DOUGLAS, University of Connecticut
*PRITCHARD, LINDA LOUISE, Oak Ridge National Laboratory
*REDFIELD, ALFRED G., IBM Watson Laboratory, Columbia University
*REUBEN, ROBERTA C., Columbia University
*SAFER, DANIEL, Brandeis University
*SCHWELITS, FAYE DOROTHY, C. F. Kettering Research Laboratory
*STOCK, GREGORY B., Johns Hopkins University
*TILNEY, LEWIS GAWTRY, University of Pennsylvania

*Wallace, Bruce Gordon, Harvard Medical School
Warden, Joseph T., University of Minnesota
*Weatherbee, James Arthur, Illinois Institute of Technology
*Weisel, John Winfield, Brandeis University
*Wolin, Edward Michael, Reed College
Wong, Wai Yan, University of Notre Dame

INVERTEBRATE ZOOLOGY

*Altalo, Mary G., Smith College
*Barish, Michael E., Massachusetts Institute of Technology
Berman, Mark S., Lawrence University
*Besso, Joseph A., Jr., University of Vermont
Blau, Helen, Harvard University
*Bremer, Karl E., University of Notre Dame
Brothers, Lynda, University of Virginia
*Burns, John R., University of Massachusetts
Cole, Tim J., University of West Florida
*Corson, David W., Jr., College of William and Mary
*Eversole, Arnold G., Syracuse University
Ferman, Johanna, University College of London
Kovacs, David A., Oregon State University
*Lam, Frank G., Oberlin College
*Lipson, Robert A., Columbia University
Loftus, Michael E., Johns Hopkins University
*Luborsky, Judith, State University of New York at Albany
*MacLeod, Murdo G., University of Glasgow
*Osman, Richard W., Brown University
Parrish, John W., Jr., Bowling Green State University
Pickvance, Simon M. J., Cambridge University
*Pittman, R. Gayle, Rice University
Ranch, Jerome P. F., De Paul University
Rieke, Carl K., Louisiana State University
*Ritzman, Roy E., University of Virginia
Sidie, James M., Jr., Indiana University
*Snider, Gilbert M., State University of New York at Stony Brook
Stein, Paul C., Southern University
Stiller, Ron A., Boston University
Stillings, Susan, Oberlin College
*Strong, Paul L., Purdue University
Stullken, Russell E., Emory University
*Sweadner, Kathy, University of California, Santa Barbara
Thompson, Stuart H., University of Washington
*Thurman, Carl L., II, University of West Florida
Tomasello, Joyce M., Case Western Reserve University
Toomey, Barbara L., Goucher College
Tosi, Laura L., Boston University
Watts, John A., Jr., Drew University
Williams, Karen, Lynchburg College

NEUROBIOLOGY

Johnson, Ernest W., University of Vermont, College of Medicine
Kalat, James W., University of Pennsylvania
Kankel, Douglas R., Brown University
Lindstrom, Jon Martin, University of California, San Diego
Macagno, Eduardo, Columbia University
Marelli, John David, University of Connecticut
Rothman, Barry S., California Institute of Technology
Steinbach, Joseph H., University of California, San Diego

4. Fellowships and Scholarships, 1970

The Bio Club:

Francine Inhaber, Botany Course

The Merkel H. Jacobs Scholarship:

Anthony Norden, Physiology Course
Donna Huang, Physiology Course

The James Watt Mavor Fund:

Murdo G. MacLeod, Invertebrate Zoology Course

5. Training Programs

Fertilization and Gamete Physiology Research Training Program

I. Instructors

Charles B. Metz, University of Miami, Program Chairman
C. R. Austin, Cambridge University, England
Giovanni Giudice, University of Palermo
Gertrude W. Hinsch, University of Miami
Kurt Köhler, University of Montpellier, France
Allen Schuetz, Johns Hopkins University

II. Consultant

Leonard Nelson, Medical College of Ohio at Toledo

III. Laboratory Assistants

Marilyn L. Cayer, Electron Microscope Assistant
Ellen Morgan, Photographic Assistant
Angela O'Rand, Secretary

IV. Trainees

Ackerman, Neil R., Stanford University
Balcuns, Astrida J., State University of New York at Albany
Baumgartel, Mona D., University of California, San Diego
Bennett, Jerry, Iowa State University
Conway, Carolyn M., University of Miami
Conway, Arthur F., University of Miami
Ewing, Richard D., Oak Ridge National Laboratory
Hull, Shirley A., Oregon State University
Kutish, Gerald F., Iowa State University
Lewis, Michael C., Rensselaer Polytechnic Institute
Merker, Jerry W., Kansas State University
O'Rand, Michael G., Temple University
Shippee, Elizabeth S., Cornell University
Tang, Frank Y., University of Toledo
Toole, Brian P., Massachusetts General Hospital
Vaughn, Jack C., Miami University, Oxford, Ohio

V. Lectures

Allen Schuetz	Hormone tissue interactions in amphibian ovarian follicles
R. Kessel	Comparative aspects of cytodifferentiation and vitellogenesis during oogenesis
G. Giudice	Ribosomal RNA synthesis in the sea urchin embryo
Wallis H. Clark, Jr.,	Spermiogenesis in *Ascaris suus*
E. L. Chambers	Effects of fertilization on ion exchange in sea urchin embryos
R. H. Barth, Jr.	The endocrine regulation of the reproduction cycle in female cockroaches

A. C. MENGE	Immunologic induction of infertility in female animals
R. STAMBAUGH	Significant enzyme in fertilization and gamete physiology
L. PIKO	Fine structural and biochemical studies of early development in sea urchins
M. J. MOSES	Macromolecular differentiations during aflagellate spermatogenesis in a coccid insect

EXCITABLE MEMBRANE PHYSIOLOGY AND BIOPHYSICS TRAINING PROGRAM

I. INSTRUCTORS

WILLIAM J. ADELMAN, JR., University of Maryland School of Medicine, Program Chairman
JOHN W. MOORE, Duke University School of Medicine
TOSHIO NARAHASHI, Duke University School of Medicine
YORAM PALTI, Hebrew University, Hadassah Medical School
WERNER R. LOEWENSTEIN, College of Physicians and Surgeons, Columbia University

II. CONSULTANTS

KENNETH S. COLE, National Institutes of Health
LORIN J. MULLINS, University of Maryland School of Medicine
DANIEL L. GILBERT, National Institutes of Health

III. TRAINEES

BASHOR, DAVID, Florida State University
BAUMANN, GILBERT, Eastern Pennsylvania Psychiatric Institute
BERLAD, ABRAHAM, State University of New York at Stony Brook
GOUDEAU, HENRI, University of Paris, France
JOHNSON, JAN, Boston University
KORDAS, MARJAN, Ljubljana University, Jugoslavia
LIPICKY, RAYMOND, University of Cincinnati School of Medicine
MONAHAN, MARCIA, Duke University School of Medicine
OOSTING, PIETER, Phillips Research Laboratories, Eindhoven, Netherlands
SCUKA, MARIA, Instituto di Fisiologia, Universita di Trieste, Italy
YAMAGUCHI, HIROSHI, Tufts University

IV. LECTURES

RICHARD D. KEYNES	Maintenance of the resting ionic concentration gradients in excitable tissues
KENNETH S. COLE	Electrical characteristics of excitable membranes
TOBIAS SCHWARTZ	The unity of classical membrane diffusion theory
	The Ussing-Teorell unidirectional flux ratio
	Osmotic phenomena and membrane pores
	The Donnan equilibrium
	The Goldman equation and its constraints
	The Goldman equation: effect of active transport
	Simple diffusion regimes and electrical equivalent circuits
GEORGE KATZ	Some aspects of instrumentation systems
	Feedback and its application
	Feedback control of membranes: methodology
JOHN W. MOORE	Voltage clamp arrangements
WILLIAM ADELMAN, JR.	Voltage clamped membrane currents in the squid axon
	Inactivation of the initial transient current
YORAM PALTI	The Hodgkin and Huxley axon model: parameter analysis for step and other command potentials
	Reconstruction of axon action potential
ROBERT E. TAYLOR	Passive cable properties in nerve
	Cable properties during nerve impulse propagation. Conduction in medullated and unmedullated axons

Toshio Narahashi	Drug action on excitable membranes I. General: conductances of endplate membranes
	Drug action on excitable membranes II. Tetrodotoxin, anesthetics and insecticides
	Drug action on excitable membranes III. Site of action and active form of anesthetics
Gerald Ehrenstein	Comparison of lipid bilayers with cell membranes
	Excitability in lipd bilayer membranes
John Reuben	Introduction to the morphology and function of the three excitable membrane components of muscle
	Electrical characteristics of the different membrane components of muscle
	The excitation contraction coupling processes
C. Ladd Prosser	Excitable membrane characteristics of smooth muscle
Darin De Lorenzo	Ultrastructure of neural membranes and their relation to sheath cells
Daniel L. Gilbert	Surface charges. I. Extension of Gouy-Chapman theory to axon membrane
	Surface charges. II. Electrokinetic determination of surface charge on cells
Ichiji Tasaki	Macromolecular approaches to the excitation process (film)
	Fluorescence studies on nerve membranes
Lorin J. Mullins	Passive electric currents and their relation to membrane currents
	Ion pumping contributions to the resting potential
	Ion membrane specificity
Fred Dodge	The myelinated axon. I. Cable properties and saltatory conduction
	The myelinated axon. II. Current-voltage relations of the Node of Ranvier
	Computations of excitation and design specifications
V. Workshops	
John Moore	Lab 8 computer simulation of axon membrane activity using a Focal program
Yoram Palti	Sigma 7 digital computer simulation of nerve behavior using a Fortran program

6. Tabular View of Attendance, 1966–1970

	1966	*1967*	*1968*	*1969*	*1970*
Investigators—Total	555	590	528	566	532
Independent	287	313	281	310	324
Library Reader	77	78	76	68	73
Research Assistants	191	199	171	188	135
Students—Total	126	132	122	118	142
Invertebrate Zoology	37	41	39	35	41
Embryology	22	20	20	20	28
Physiology	29	31	30	30	31
Experimental Botany	18	20	15	16	19
Ecology	20	20	18	17	23
Trainees—Total	16	16	17	29	33
Total Attendance	710	738	667	708	707
Less persons represented in two categories	0	4	7	5	0
	710	734	660	703	707
Institutions Represented—Total	198	177	169	187	191
Foreign Institutions Represented	28	29	23	24	21

7. Institutions Represented, 1970

Agnes Scott College
Alabama, University of
Albany Medical School
Albert Einstein College of Medicine
Allegheny General Hospital
American Museum of Natural History
Amherst College
Arizona, University of
Arizona, University of, College of Medicine
Augustana College
Bard College
Boston City Hospital
Boston College
Boston University
Bowling Green State University
Brandeis University
Brookhaven National Laboratory
Brooklyn College, The City University of New York
Brown University
Bryn Mawr College
California Institute of Technology
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, Santa Barbara
California, University of, Santa Cruz
Carnegie Institution of Washington
Case Western Reserve University
Central Connecticut State College
Chatham College
Chicago, University of
Chicago Lying-in Hospital
Cincinnati, University of
City College of New York, The
Colby College
Cold Spring Harbor Laboratory
College of Osteopathic Medicine and Surgery
College of William and Mary
Colorado, University of, Medical Center
Columbia University
Columbia University, College of Physicians and Surgeons
Connecticut, University of
Connecticut, University of, Health Center
Connecticut, University of, Medical School
Cornell University
Cornell University Medical College
Dartmouth College
Dartmouth Medical School
Davidson College
Delaware, University of
De Paul University
Drew University
Duke University
Duke University Medical Center
Eastern Pennsylvania Psychiatrist Institute
Emory University
Eye Research Foundation of Bethesda
Florida Atlantic University
Florida, University of
Florida State University
Goucher College
Harvard Medical School
Harvard University
Hawaii, University of
Houston, University of
Hudson Valley Community College
Hunter College
Illinois, University of
Illinois Institute of Technology
Indiana University
Indiana University Medical School
Institute for Basic Research in Mental Retardation
Institute for Cancer Research, The
Institute for Muscle Research, The
Institute of Molecular Evolution, University of Miami
Iowa, University of
Iowa State University
Jersey City State College
John Carroll University
Johns Hopkins University, The
Johns Hopkins University, The, Hospital
Johns Hopkins University, The, School of Hygiene
Johns Hopkins University, The, School of Medicine
Juniata College
Kansas, University of
Kansas State University
Kentucky, University of
Kettering, Charles F., Research Laboratory
Lawrence University
Lehman College
Louisiana State University
Lynchburg College
Maine, University of
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts, University of
Massachusetts Audubon Society
Massachusetts General Hospital
Massachusetts Institute of Technology
Medical College of Georgia
Medical College of Ohio at Toledo
Mellon Institute of the Carnegie-Mellon University

Miami, University of
Miami University
Michigan, University of
Middlebury College
Minnesota, University of
Minnesota, University of, School of Medicine
Missouri, University of
Mount Holyoke College
National Biomedical Research Foundation
National Institute of Mental Health
National Institutes of Health
New Mexico, University of, School of Medicine
New York Blood Center, The
New York University
New York University College of Dentistry
New York University Medical College
North Carolina, University of
North Carolina State University, Raleigh
Northwestern University
Notre Dame, University of
Oak Ridge National Laboratory
Oberlin College
Ohio Dominican College
Ohio Wesleyan University
Ohio University
Oklahoma, University of
Oregon, University of
Oregon State University
Pennsylvania, University of
Pennsylvania, Univeristyof, School of Medicine
Pittsburgh, University of
Pomona College
Princeton University
Purdue University
Queens College, The City University of New York
Reed College
Rensselaer Polytechnic Institute
Rhode Island, University of
Rhode Island Hospital
Rice University
Rochester, University of
Rochester, University of, Medical School
Rockefeller University, The
Rutgers University
Rutgers University Medical School
St. Louis University
St. Vincent College
Scripps Institution of Oceanography
Seton Hill College
Smith College
South University
Stanford University
State University of New York, Downstate Medical Center
State University of New York, Upstate Medical Center
State University of New York at Albany
State University of New York at Buffalo
State University of New York at Stony Brook
State University of New York at Syracuse
Syracuse University
Temple University
Tennessee, University of
Texas Technical University
Texas, University of, Austin
Toledo, University of
Trinity College
Tufts University
Tulane University
Vanderbilt University School of Medicine
Vassar College
Vermont, University of
Vermont, University of, School of Medicine
Veterans Administration Central Office, Washington. D. C.
Veterans Administration Hospital, Brooklyn
Veterans Administration Hospital, Pittsburgh
Virginia, University of
Virginia, University of, School of Medicine
Washington University
Washington University School of Medicine
Wesleyan University
West Florida, University of
Wisconsin, University of
Wistar Institute of Anatomy and Biology
Woods Hole Oceanographic Institution
Yale University
Yale University School of Medicine

FOREIGN INSTITUTIONS REPRESENTED, 1970

Calgary, University of Canada
Cambridge, University of, England
Centre National de la Recherche Scientifique, France
Glasgow, University of, Scotland
Hadassah School of Medicine, Israel
Hebrew University Medical School, Jerusalem
Hiroshima University School of Medicine, Japan
Institute of Pathophysicology, Yugoslavia
Institute of Physiology, Trieste
International Institute of Genetics and Biophysics, Italy
Leeds, University of, England
McGill University, Canada
Medical Research Council, England
Montreal, University of, Canada
Ottawa, University of, Canada
Oxford, University of, England
Palermo, University of, Italy
Paris, University of, France
Research Institute of National Defence, Sweden
Tel-Aviv University, Israel
Universidad Central de Venezuela
University College, London

8. Friday Evening Lectures, 1970

July 3	John Dowling Johns Hopkins University Medical School	The vertebrate retina: An approachable piece of brain
July 10	Malcolm Steinberg Princeton University	How cells self-assemble into tissues and organs
July 17	Joel Rosenbaum Yale University	Synthesis and assembly of flagellar microtubules
July 23	L. Tauc National Scientific Research Center, Paris Alexander Forbes Lecturer at MBL	Postsynaptic action of transmitter substances
July 24	L. Tauc	Long lasting modifications of synaptic efficacy
July 31	Moshe Shilo Hebrew University	*Bdellovibrio* as a model in the understanding of intracellular parasitism
August 7	Walter Gilbert Harvard University	Repressors and operators
August 14	Donald D. Brown Carnegie Institution	An analysis of ribosomal genes in development
August 21	Roger Payne The Rockefeller University	"Songs" of humpback whales

9. Tuesday Evening Seminars, 1970

July 14	C. R. Austin R. G. Edwards B. D. Bavister R. L. Gardner	Initiation of development *in vitro* in the hamster and in man
	R. L. Gardner	Manipulative experiments on the mammalian blastocyst
	W. D. Russell-Hunter Albert J. Burky R. Douglas Hunter	Interpopulation variation in shell components in the stream limpet, *Ferrissia*
	Roger Milkman	A fundamental error in the general model of genetic selection
	Catherine Henley	Ultrastructure of the negatively stained spermatozoon of the earthworm

July 21

Bryan P. Toole	Hyaluronic acid and the early blastema of the regenerating newt limb
Eric J. Simon	Inhibition of RNA phage reproduction and macromolecular synthesis
Max Braverman	Regulation of hydranth formation in the colonial hydroid, *Podocoryne carnea*
Ruth Sager	Genetic circularity of an organelle DNA in *Chlamydomonas*

July 28

M. E. Spira I. Parnas F. Bergmann	Exitatory and inhibitory regulation of efferent nerve activity in the phallic nerve of the cockroach *Periplaneta americana* (L)
H. Ripps J. Dowling	Electrical and photochemical signs of adaptation in the skate retina
D. Landowne	The role of the sodium pump in adaptation in the frog muscle spindle
W. H. Clark, Jr G. W. Hinsch	Ultrastructural study of the secondary septa of *Metridium* sp.

August 4

N. W. Cornell	Metabolic controls and biological variation
M. Fingerman K. R. Rao	Analysis of the color changes induced by serotonin (5-hydroxytryptamine) and lysergic acid diethylamide (LSD) in the fiddler crab, *Uca pugilator*
F. C. G. Hoskin	Enzymatic hydrolysis of nerve gases in relation to function
W. R. Kem	Chemistry and biology of nemertine neurotoxins

August 11

G. Weissmann	Mechanisms of enzyme release from natural and artificial lysosomes
S. Zigman	Isoelectric focusing of lens gamma crystallins
W. D. Sullivan, S. J.	Microtubules in the macronucleus of *Tetrahymena pyriformis* Gl.
G. W. Hinsch	Some factors controlling reproduction in *Libinia emarginata*

August 18

D. B. Wilson	On histocompatibility antigens
H. Gewurz Vanessa Birdsey Donald Johnson Jean Lindorfer Kay Townsend Anita Gewurz	An inducible lysin in *Limulus* with similarities to the complement system of vertebrates
H. T. Epstein	Enzyme changes associated with development of bacterial competence
R. A. Prendergast	Mammalian macrophage activating factor from the sea star *Asterias forbesi*

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Including Action of 1970 Annual Meeting

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V. REPORT OF THE LIBRARIAN

During the summer the library committee sent questionnaires to summer investigators asking for help in selecting and recommending books, monographs and journals in their specific fields. Seventy-two people volunteered their services and they were divided into 18 subject panels. Comprehensive lists were worked on by each group and sent to the library. Unfortunately, due to the present state of the economy, the library budget does not call for any new book purchases; however, the lists establish an excellent basis for library needs in the book section and will be of value when money is available.

A subscription cost study for periodicals was made and we found that 651 of the periodicals raised their rates in the past five years. In 1965 subscriptions to these journals totaled $15,095 and in 1970 the same titles totaled $29,014, nearly a 100% increase. We definitely do not want to cancel or weed out journal subscriptions; therefore, the book section must suffer until the budget can be expanded.

In the fall we gave permission to the G. K. Hall Publishing Company of Boston to reproduce our card catalog of authors. An estimated 354,000 cards will be reproduced in twelve volumes. The final volume, containing our journals catalog, will be sold separately. Prepublication price of the complete catalog will be $790 in the United States. The Library will receive royalties after a certain number of sets have been sold.

In 1970 we received 4,300 interlibrary loan requests for articles here at MBL and we made 330 requests of other libraries for material we did not have. Ninety-three percent of our interlibrary loan activity involved outside libraries making requests of our collection. 2,054 volumes were sent to the bindery and our holdings now total 143,658 volumes. This does not include the reprint collection of 250,000 papers.

Total number of serial titles		4,106
Number received currently		2,436
(On subscription	988	
On exchange	991	
On gift basis	377)	
Number of textbooks added		300
(Received from book exhibit	215)	
(No reprints were added—see 1969 report)		

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1970, amounted to $2,173,414 and the corresponding securities are entered in the books at a value of $1,573,362. This compares with values of $2,197.603 and $1,574,735, respectively, at the end of the preceding year. The average yield on the securities was 3.99% of the market value and 5.51% of the book value. Uninvested principal cash was $2,641. Classification of the securities held in the Endowment Fund appears in the Auditor's Summary of Investments.

The market value of the Pooled Securities at December 31, 1970, amounted to $770,487 as compared to book values of $662,428. These figures compare with values of $763,700 and $634,152, respectively, at the close of the preceding year. The average yield on the securities was 3.71% of the market value and 4.32% of the book value. Uninvested principal cash was in the amount of $485.

The proportionate interest in the Pool Fund Account of the various funds, as of December 31, 1970, is as follows:

Pension Funds	25.524%
General Laboratory Investment	19.994%
F. R. Lillie Memorial Fund	2.180%
Anonymous Gift	.748%

Other:

Bio Club Scholarship Fund	.568%
Rev. Arsenius Boyer Scholarship Fund	.686%
Gary N. Calkins Fund	.649%
Allen R. Memhard Fund	.124%
Lucretia Crocker Fund	2.364%
E. G. Conklin Fund	.397%
Jewett Memorial Fund	.204%
M. H. Jacobs Scholarship Fund	.284%
Herbert W. Rand Fellowship	20.165%
Mellon Foundation	9.518%
Mary Rogick Fund	2.087%
Swope Foundation	5.238%
Clowes Fund	9.270%

Donations from MBL Associates for 1970 amounted to $9,724 as compared with $9,246 for 1969. Unrestricted gifts from foundations, societies and companies amounted to $20,008.

During the year we administered the following grants and contracts:

Investigators	*Training*	*MBL Institutional*
6 NIH	3 NIH	3 NIH
3 NSF	2 NSF	1 AEC
1 NIMH		
2 ONR		
1 WHIH		1 Ford
13	5	5

Overhead rates of 20% or 25% of allowable direct costs continued in effect during 1970 for several research grants and contracts which had been awarded prior to August 1, 1969. Research grants and contracts initiated after that date provided for indirect costs on a square foot basis, based on the assigned space for a particular research project. A provisional rate, effective January 1, 1970 of $10.00 per square foot is presently being used.

The following is a statement of the Auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts

We have examined the balance sheet of Marine Biological Laboratory as at December 31, 1970, the related statement of operating expenditures and income and statement of funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We examined and have reported on financial statements of the Laboratory for the year ended December 31, 1969.

In our opinion, the accompanying financial statements (pages 84 to 88) present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1970 and 1969 and the results of its operations for the years then ended on a consistent basis except for the change, in which we concur, referred to in note A.

The supplementary schedules (pages 89 and 90) included in this report were obtained from the Laboratory's records in the course of our examination and, in our opinion, are fairly stated in all material respects in relation to the financial statements, taken as a whole.

Boston, Massachusetts
March 24, 1971

LYBRAND, ROSS BROS. AND MONTGOMERY

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1970 and 1969

Investments

	1970	*1969*
Investments held by Trustee:		
Securities, at cost (approximate market quotation 1970—$2,173,414)	$ 1,573,362	$1,574,735
Cash	2,641	1,104
	$ 1,576,003	$1,575,839
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation 1970—$770,487) less $5,728 temporary investment of current fund cash	656,700	628,424
Other investments	1,125,150	1,474,150
Cash	485	447
Accounts receivable		33
Due from current fund	65,176	61,622
	$ 3,423,514	$3,740,515

Plant Assets

	1970	*1969*
Land, buildings, library and equipment	9,662,611	6,072,007
Less allowance for depreciation (note A)	1,899,406	1,802,581
	7,763,205	4,269,426
Construction in progress (note B)	2,476,261	3,758,341
Investments at cost (approximate market quotation 1970—$536,875)	712,745	707,327
Due from current funds		13,401
	$10,952,211	$8,748,495

Current Assets

	1970	*1969*
Cash	276,166	163,471
Temporary investment in pooled securities	5,728	5,728
Accounts receivable (U. S. Government, 1970—$52,621; 1969—$52,391)	147,881	137,757
Inventories of supplies and bulletins	41,749	45,593
Other assets	10,310	6,619
Due to plant funds		(13,401)
Due to endowment funds	(65,176)	(61,622)
	$ 416,658	$ 284,145

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1970 and 1969

Invested Funds

	1970	*1969*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$ 1,576,003	$1,575,839
Endowment funds for awards and scholarships:		
Principal	427,702	427,663
Unexpended income	51,458	44,630
	479,160	472,293
Unrestricted funds functioning as endowment	1,179,190	1,528,190
Retirement fund	246,833	217,433
Pooled investments—accumulated loss	(57,672)	(53,240)
	$ 3,423,514	$3,740,515

Plant Funds

Funds expended for plant, less retirements	11,797,632	9,598,348
Less allowance for depreciation charged thereto	1,899,406	1,802,581
	9,898,226	7,795,767
Accounts payable	341,240	240,001
Unexpended plant funds	712,745	712,727
	$10,952,211	$8,748,495

Current Liabilities and Funds

Accounts payable and accrued expenses	10,309	9,526
Advance subscriptions	34,275	30,141
Unexpended grants—research	66,324	80,811
Unexpended balances of gifts for designated purposes	25,020	23,278
Current fund	280,730	140,389
	$ 416,658	$ 284,145

The accompanying notes are an integral part of the financial statements.

Note A—During the current year the Laboratory changed its practice from providing depreciation in the year following the acquisition of a plant asset to the month following acquisition. This change had the effect of increasing depreciation expense for 1970 by approximately $46,000.

The Laboratory provides for reduction of book amounts of plant assets at annual rates ranging from 1% to 5% of the original cost of the assets.

Note B—The Laboratory has commitments of approximately $180,000 for the completion of construction of a dormitory dining-hall.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF OPERATING EXPENDITURES AND INCOME

Years Ended December 31, 1970 and 1969

	1970						
	Salaries and Wages	*Other Costs and Expenses*	*Depreciation* (Note A)	*Total*	*Charged to Grants*	*Total*	*1969*
Operating Expenditures							
Instruction		$ 32,697	$ 37,196	$ 69,893	$202,520	$ 272,413	$ 208,859
Research		32,400	75,113	107,513	372,457	479,970	460,404
Dormitories	$ 20,585	43,027	29,907	93,519		93,519	91,904
Dining	21,815	42,846	317	64,978		64,978	61,933
Library	33,844	13,060	18,030	64,934		64,934	64,903
Back sets, serials and binding		43,882		43,882		43,882	42,186
Biological Bulletin	5,066	36,058		41,124		41,124	46,000
Support services:							
Apparatus	48,468	32,435		80,903		80,903	104,731
Supply	87,449	57,013	4,507	148,969		148,969	151,652
Administration	91,163	73,996		165,159		165,159	159,596
Plant operation	153,643	114,803	2,353	270,799		270,799	230,590
Grant expenditures for support services					47,483	47,483	48,875
Other		33,357		33,357		33,357	31,800
	$462,033	$555,574	$167,423	1,185,030	$622,460	1,807,490	1,703,433

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF OPERATING EXPENDITURES AND INCOME

Years Ended December 31, 1970 and 1969

	1970					
	Fees	*Other*	*Total*	*Charged to Grants*	*Total*	*1969*
Income						
Instruction	$ 48,600		48,600	202,520	251,120	227,217
Research	159,889		159,889	372,457	532,346	497,562
Dormitories		$ 99,633	99,633		99,633	83,640
Dining		78,828	78,828		78,828	71,938
Library	17,125	33,078	50,203		50,203	44,508
Biological Bulletin		54,715	54,715		54,715	39,789
Support services:						
Apparatus		36,592	36,592		36,592	40,378
Supply		62,379	62,379		62,379	54,762
Administration		16,101	16,101		16,101	15,336
Investments income		183,787	183,787		183,787	230,225
Gifts used for current expense		38,690	38,690		38,690	34,379
Allowance for indirect costs		62,666	62,666		62,666	74,958
Grants for general support		48,103	48,103		48,103	83,911
Grants for support services				47,483	47,483	48,875
Other		1,925	1,925		1,925	3,658
	$225,614	$716,497	942,111	$622,460	1,564,571	1,551,136
Excess of current expenditures and depreciation over current income			242,919		242,919	152,297
Reduction in plant funds for depreciation			167,423		167,423	127,175
Excess current expenditures			$ 75,496		$ 75,496	$ 25,122

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year Ended December 31, 1970

	Balance December 31, 1969	*Gifts and Other Receipts*	*Invest-ment Income*	*Used for Current Expenses*	*Other Expendi-tures*	*Balance December 31, 1970*
Invested funds	$3,740,515	$ 33,225 (349,000) (2)	$205,579	$183,550	$ 23,255	$3,423,514
Unexpended plant funds	$ 712,727	2,204,064	23,873		2,227,919	$ 712,745
Unexpended research grants	$ 80,811	718,741		733,228		$ 66,324
Unexpended gifts for designated purposes	$ 23,278	11,866		9,724	400	$ 25,020
Current fund	$ 140,389	(75,496) (1) 349,000 (2)			133,163	$ 280,730
		$2,892,400	$229,452	$926,502	$2,384,737	
Gifts and grants for facilities construction		2,204,064				
Other gifts and receipts		11,866				
Grants for research, training and support		718,741				
Appropriated from current income and other		33,225				
(1) Excess of current expenditures over income		(75,496)				
(2) Transfer from invested funds						
		$2,892,400				
Expended for new laboratory and dormitory—dining hall					2,361,082	
Scholarship awards					7,766	
Payments to pensioners					11,057	
Loss on sale of securities					4,432	
Other					400	
					$2,384,737	

MARINE BIOLOGICAL LABORATORY

Summary of Investments

December 31, 1970

	Cost	Per Cent of Total	Market Quotations	Per Cent of Total	Investment Income 1970
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	$ 25,065	2.0	$ 26,175	1.5	$ 1,250
Corporate bonds	718,227	57.6	536,098	30.2	31,937
Preferred stocks	84,770	6.8	62,449	3.5	3,385
Common stocks	419,448	33.6	1,149,489	64.8	35,888
	1,247,510	100.0	1,774,211	100.0	72,460
General educational board endowment fund:					
U. S. Government securities	51,113	15.7	53,397	13.3	2,601
Other bonds	178,883	54.9	135,603	34.0	7,502
Preferred stocks	15,476	4.7	9,177	2.3	580
Common stocks	80,380	24.7	201,026	50.4	3,586
	325,852	100.0	399,203	100.0	14,269
Total securities held by Trustee	$1,573,362		$2,173,414		86,729
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	93,557	14.1	93,493	12.1	1,259
Corporate bonds	163,356	24.7	134,675	17.5	9,854
Preferred stocks	60,157	9.1	56,025	7.3	1,120
Common stocks	345,358	52.1	486,294	63.1	16,357
	662,428	100.0	$ 770,487	100.0	28,590
Less temporary investment of current fund cash	5,728				237
	656,700				28,353
Other investments:					
U. S. Government securities	27,938				1,133
Other bonds	15,029				750
Common stocks	49,634				2,714
Real estate	17,549				
Short-term commercial notes	1,015,000				92,898
	1,125,150				97,495
Total investments of other endowment and unrestricted funds	$1,781,850				125,848

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1970

	Cost	*Investment Income 1970*
Total		212,577
Custodian's fees charged thereto		6,998
Investment income distributed to invested funds		205,579
Plant investments:		
Federal agency and corporate bonds	140,000	5,945
Common stock	569,874	17,824
Preferred stock	2,871	104
	$ 712,745	23,873
Current investments:		
Temporary investment in pooled securities	$ 5,728	237
Total investment income		$229,689

Reference: *Biol. Bull.*, **141**: 91–98. (August, 1971)

CELLULAR RESPONSES TO FOREIGN BODIES IN THE TUNICATE *MOLGULA MANHATTENSIS* (DeKay)[1]

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Much is known about the cellular and immunoglobulin responses of vertebrates to foreign substances; however, such information on the various invertebrate phyla is incomplete. Tunicates are primitive chordates in which a dorsal nerve cord and notochord are present only in a short-lived larval form. While there is an early reference to a humoral antibacterial agent in tunicates (Cantacuzène, 1919), no data is available on the cellular defense mechanisms of these animals. This paper describes the encapsulation of large foreign bodies by hemocytes, the phagocytosis and elimination of injected particulate dyes, and the cellular response to tunicate tissue surgically implanted in *Molgula manhattensis.*

The blood cell types characteristic of *M. manhattensis* are similar to those of *Phallusia mammillata* described by Endean (1960). The following cell types comprise the majority of the circulating blood cells; vanadocytes, signet ring cells, and amoebocytes. Vanadocytes are characterized by the presence of a vanadium-protein complex (hemovanadin) associated with sulfuric acid which possibly serves to maintain its natural oxidation state (Webb, 1939). Hemovanadin has strong reducing properties, but it is not a respiratory pigment (Barrington, 1965). Endean (1961) using electron microscopy observed the formation of cellulose by vanadocytes in the tunic of *P. mammillata.* Vanadocytes are 7–8.5 μ in diameter with a central nucleus surrounded by green refractile bodies containing hemovanadin. The cells are strongly acidic in their staining reactions and are undoubtedly of the "green-cell type" described in *M. manhattensis* by George (1939). Signet ring cells are 8–10 μ in diameter and contain a large vacuole (usually containing clear material) which presses the nucleus against the plasma membrane compressing the cytoplasm into a thin peripheral band. Compartment cells average 8.5 μ in diameter, their cytoplasm is divided into a number of chambers by an internal membrane system. Compartment cells frequently elongate and become amoeboid. Amoebocytes are variable in size, averaging 10–15 μ in length and 5–8 μ in width. Both granular and hyaline amoebocytes are present in tunicate blood.

MATERIALS AND METHODS

Adult specimens of *M. manhattensis* were collected at the marine biology facilities of the University of Delaware at Lewes, Delaware. The animals were maintained in polyethylene tanks in aerated sea water at 15° C.

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[1] This work was supported by a National Science Foundation Traineeship.

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To evaluate cellular responses to large, inert objects, 2–4 cm long fragments of broken glass coverslips were inserted into the tissues of the wall of the branchial sac. The branchial sac is a perforated baglike structure which functions in both filter-feeding and respiration. It is richly supplied with blood vessels and sinuses so that circulating blood cells come into direct contact with the glass. If the glass fragments were inserted carefully, the experimental animals survived as long as controls under laboratory conditions. The optimal location for insertion of glass fragments was found to be high on the lateral surface near the siphons, avoiding the large dorsal and ventral blood vessels and the major organs. The glass fragments were periodically excised, bathed in sea water, and examined under a phase-contrast microscope. Permanent mounts were prepared by fixing the blood cells with Bouin's solution, staining with hematoxylin and eosin, clearing with xyene, and mounting in Permount (Fisher Scientific Co., Philadelphia, Pennsylvania).

One per cent solutions of trypan blue or carmine were injected intracardially after a portion of the tunic had been sliced away to facilitate visualization of the heart. The animals were sacrificed at various intervals after injection. The tunic was removed, the animals fixed in Bouin's solution, dehydrated, cleared with xylene, embedded in paraffin, and sectioned at 10 μ. The sections were placed on slides, dewaxed, stained (trypan blue-injected animals with eosin, and carmine-injected animals with hematoxylin), cleared, and mounted with Permount.

Grafts of tunicate tissue were implanted into the wall of the branchial sac of recipient animals. Experimental animals received either autografts (tissue excised from another area of their own branchial sac) or allografts (tissue excised from the branchial sac of another tunicate). The following technique was used for the implantation of both autografts and allografts. Minute (2–4 mm) pieces of branchial sac tissue were excised and stained in a solution of 5% trypan blue for 2 minutes to facilitate subsequent location of the graft. Neither graft nor host was treated with antibiotics because bacterial infection arising from surgery was never observed in serial sections of the graft and surrounding tissue. The host's tunic was punctured with a 10 gauge needle in the lateral region below the siphons. The points of a pair of fine, curved forceps were inserted and spread to enlarge the opening in the tunic. A small cut was made in the outer portion of the wall of the branchial sac. The graft was implanted into this cut using a sterile stainless steel wire so that it rested in a surgically prepared pocket within the wall of the branchial sac. The opening in the tunic closed within 3 days in those animals surviving the operation. At the termination of the experiment, the tunic was removed and the entire region of the graft was excised and fixed in Bouin's solution. Specimens were prepared for histological examination in the same fashion as the dye-injected animals.

Hematoxylin and eosin-stained serial sections of entire untreated tunicates were used to study the histology of control animals. These preparations were used as a basis of comparison for evaluating cellular responses initiated by the various experimental manipulations.

A hemocytometer was employed to determine the total and differential blood cell count of cardiac blood samples from 10 adult tunicates. The total and differential hemocyte counts in branchial sac tissue, grafts, and on glass fragments were obtained by averaging the numbers of cells counted on at least 5 different microscopic fields in the region in question.

Results

I. Cellular reactions to glass fragments

Hemocytes were rarely observed on the portions of the glass fragments which were either outside the animal or in the lumen of the branchial sac; however, the portions which were within the walls of the branchial sac were encapsulated by vanadocyte-mediated reactions in all of 60 experimental animals. The cellular response was immediate, many blood cells covered the glass within 30 minutes.

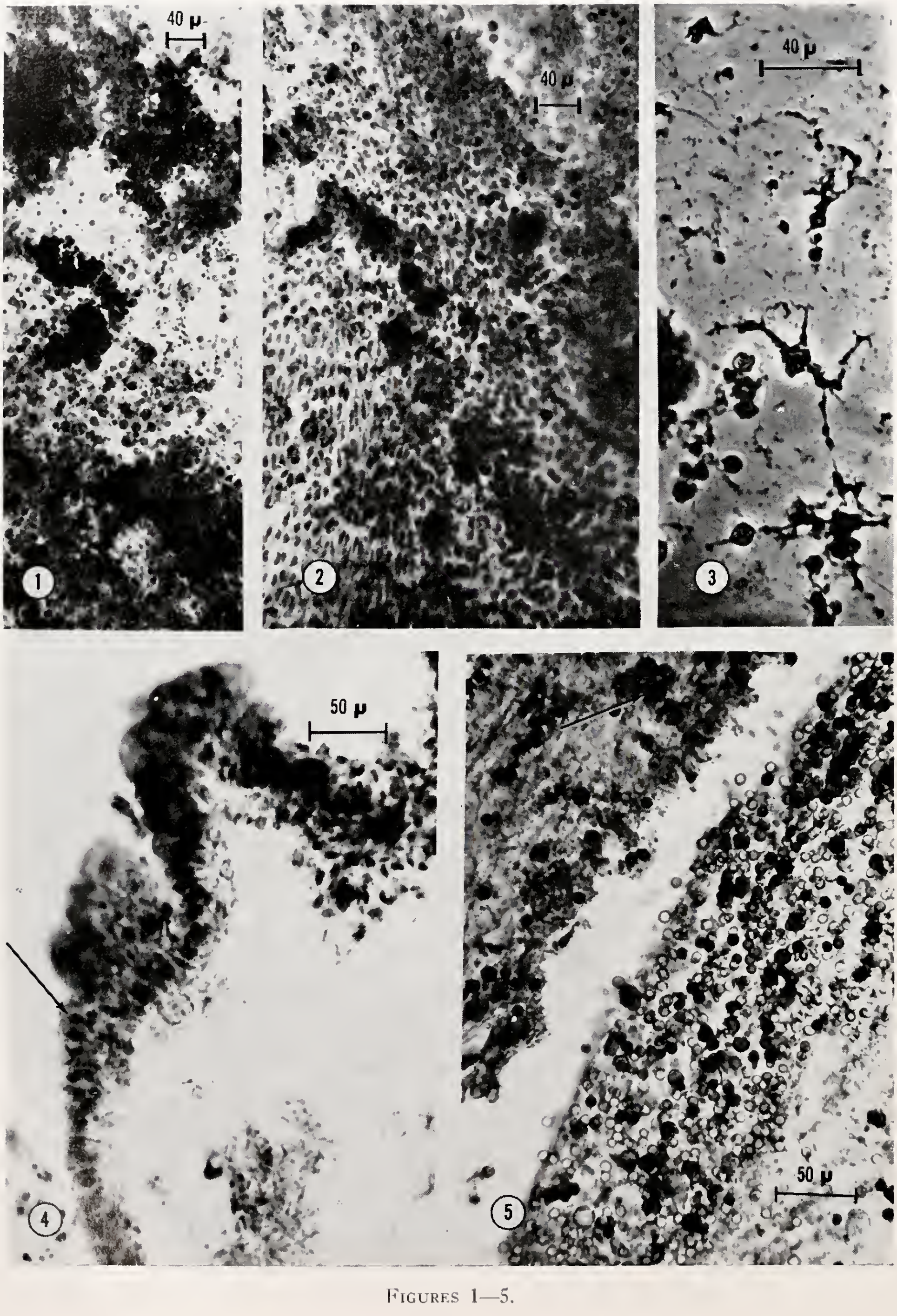
The mean number of cells per mm^3 of *M. manhattensis* blood was 20,800 (range: 17,200–24,600 for 10 adults). These cells were composed of about 40% amoebocytes, 30% vanadocytes, 15% signet ring cells, 5% compartment cells, and 10% other cell types.

One day after insertion, vanadocytes and signet ring cells were present in about equal numbers and together consituted more than 95% of the hemocytes coating the glass. Two days post-injection, at least 90% of the blood cells adhering to the glass were vanadocytes, the rest being signet ring cells. Vanadocytes (or vanadocyte-derived cells) accounted for essentially all of the cells observed on glass fragments which had been within branchial sac tissue 3 days or longer. The difference between the hemocyte composition of the cell aggregates on the glass and the cellular make-up of the circulating blood indicates that only specific cell types participate in this foreign body response.

The most commonly encountered capsules surrounding glass fragments were composed of multilayered vanadocyte aggregates (Fig. 1). The clusters were often 8–20 cell layers thick and completely covered the foreign body. Capsules withdrawn after 40 days within the branchial sac wall were not appreciably thicker than those removed after 10 days, and were composed of morphologically typical vanadocytes.

In some areas the vanadocyte aggregates did not continue to expand and thicken after 2–3 days. Individual cells appeared to separate and radiate from the periphery of the aggregates. These hyaline (or slightly granular) amoeboid cells were about 4×9 μ in size with few pseudopodia and a central nucleus. The cells became oriented so that their long axes were roughly parallel and formed monolayers of cuboidal or columnar cells (Fig. 2). In some preparations the cells seemed to be separated from each other by a space of about 1 μ. Phase contrast microscopy revealed that the cells were in contact; however, the plasma membrane is thin and a band of clear cytoplasm is directly beneath the membrane. These cells were morphologically distinct from blood cells or tissue cells of untreated tunicates. Vanadocytes did not aggregate on the cells of these monolayers.

The vanadocytes adhering to the surface of the glass were active in producing tunic matrix material (tunicin). Tunicin production was greatest in the region of contact between the glass and the tunic; however, it was formed also on the portion of the glass within the wall of the branchial sac. Vanadocytes were loosely connected to each other by strands of tunicin as early as 3 days (Fig. 3), but the production of tunic material proceeded slowly. One week was required for the


40 μ
1
40 μ
2
40 μ
3
50 μ
4
5
50 μ

FIGURES 1—5.

development of sheets of tunicin upon the glass. Within 2–6 hrs after insertion of a glass fragment, blood cells from the epidermis and branchial sac coagulated around the glass at its point of entrance into the tunic. The tunic was repaired, as described above, resulting in a tight fusion around the glass. Capsules examined after a week or more within the walls of the branchial sac usually were partially composed of tunicin strands.

II. Injection of dyes

The fate of trypan blue and carmine was determined after intracardial injection into *M. manhattensis.* Trypan blue flocculated immediately (because of chelation with divalent cations such as Mg^{++} and Ca^{++} present in tunicate blood), while carmine remained colloidal. Hence it was possible to study the phagocytosis of particles of different sizes and different chemical compositions. Carmine was injected into 25 adult *M. manhattensis,* 50 received intracardiac injections of trypan blue.

Carmine particles were phagocytosed readily and were visible in minute vacuoles in the cytoplasm of amoebocytes by 2–3 hours. No free particulate carmine was observed in the blood 1 day after injection, presumably as a result of the action of the amoebocytes. Carmine-bearing amoebocytes (and a few signet ring cells) could be identified for 7–10 days following injection of the dye. No carmine was observed within blood cells examined 2 weeks or more after injection. Microscopic examination of serial sections of carmine-injected animals failed to reveal any sites of dye deposition within tissues other than hemocytes.

Trypan blue flocculated after injection to form masses ranging from 50–100 μ in diameter. Phagocytic activity was seen at the periphery of the clumps of dye within a few hours. Groups of vanadocytes were clustered around the massses within 2–3 days; however, no encapsulation of trypan blue was observed. Trypan blue could be identified in granules in amoebocytes and in vacuoles of signet ring cells. Microscopic examination of stained and unstained serial sections revealed the presence of trypan blue in the ciliated epithelial cells of the branchial sac as early as 6 hrs after intracardial injection. The concentration of the dye in these cells increased until the majority contained numerous trypan-blue filled vesicles (Fig. 4). Intracellular dye concentration was greatest on the third day after injection, after which time it gradually disappeared from the branchial sac

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FIGURE 1. Partial encapsulation of a glass fragment 2 days after insertion into branchial sac tissue. The formation of multilayered vanadocyte aggregates may be seen in several areas; hematoxylin and eosin.

FIGURE 2. Formation of a cellular monolayer (left) from a vanadocyte aggregate (upper right) on a glass fragment 4 days after insertion into the wall of the branchial sac; hematoxylin and eosin.

FIGURE 3. Phase contrast microscopy of vanadocytes connected to each other by strands of tunic material on a glass fragment after being in the wall of the branchial sac 3 days.

FIGURE 4. Trypan blue (arrow) in the epithelial cells of the branchial sac 3 days after intracardial injection; hematoxylin and eosin.

FIGURE 5. Juncture of autograft (lower right) and host tissue (upper left) 7 days after implantation; hematoxylin and eosin. Vanadocytes are present in the mucus which separates graft from host and the graft is heavily infiltrated by vanadocytes. Large, atypical blood cells (arrow) associated with the graft reaction are clustered in the host's branchial sac.

epithelium. Trypan blue was usually most concentrated in the area of the nucleus. The dye also was observed in the cells of the gut epithelium. Little or no trypan blue was found in either the blood or tissues of the tunicates 6 days after injection.

III. Tissue gratfing

Thirty-five branchial sac autografts and 30 allografts were performed. In neither series of experiments were any grafts even temporarily fused with the host tissue. The grafts were separated from th host's branchial sac by a mucus-filled space of about 20–70 μ. Blood cells were often observed within the mucus indicating that it presented no barrier to their migration. However the mucus, which was released liberally in the region of the most minute wound, precluded the intimate contact between graft and host which is essential for even temporary union.

Marked cellular response to the implanted tissues were usually observed. The morphology and blood cell composition of both graft and host branchial sac were normal for the first 2 days. During the third day the numbers of signet ring cells and vanadocytes in the graft and surrounding tissues became slightly elevated. After day 4 the graft contained a concentration of vanadocytes (cells per unit area of section) at least 3 times that present at the time of implantation. Seven days after implantation the graft was completely infiltrated with vanadocytes which totally masked the morphology of the graft (Fig. 5). Often a number of large cells (30 μ in diameter) were seen in the tissues near the graft. These cells did not resemble any blood cell type encountered in non-grafted animals. These cells were similar in size to the giant cells associated with inflammatory reactions in the annelids *Lumbricus terrestris* and *Eisenia foetida* described by Cooper (1969); however, they did not appear to be polynucleated.

The blood cells within the grafts lost their typical morphology and eventually ruptured. Twelve to 14 days after implantation the grafts were necrotic masses. Encapsulation of the grafts by hemocytes was not observed.

DISCUSSION

The results of this study indicate that *Molgula manhattensis* has the capacity to recognize foreign bodies of various kinds and responds to them with a number of cellular reactions. The vanadocyte plays a central role in these responses, a property not previously associated with this hemocyte.

Encapsulation of foreign objects has been reported in many organisms; usually the capsules are formed by the accumulation of blood cells around the object, followed by gradual fibrotic alterations of the cells. In *M. manhattensis* three vanadocyte-dependent encapsulation mechanisms were observed in response to the insertion of glass fragments into the branchial sac. Any or all of these mechanisms may operate in the production of any given capsule. The most commonly observed encapsulation process involved the production of a many-layered structure composed of vanadocytes. The morphology of individual vanadocytes within the capsule was not different from that of circulating vanadocytes. Portions of the capsule may be made of monolayers of vanadocyte-derived cells which are formed by the differentiation of amoebocytic cells arising from the periphery of vanadocyte aggregates. In no case were these amoebocytes seen to move across already established monolayers and no vanadocyte aggregates formed over the monolayers. In older capsules (3 weeks +) these monolayers were rarely observed, this may indicate that they are labile structures which are eventually replaced by vanadocyte clusters. It is possible that circulating vanadocytes are composed of several populations of morphologically indistinguishable cells with different potentialites, some of which are capable of monolayer formation. Signet ring cells, which adhere to the glass during the first several days, may transform into vanadocytes according to Endean's (1960) scheme. Possibly vanadocytes formed in this way have slightly different characteristics from circulating vanadocytes. Perhaps some chemical or structural differences exist from place to place on the surface of the glass which influence the differentiation of the adhering cells. Other portions of the capsule were composed of sheets of tunic material which were probably synthesized by vanadocytes.

The encapsulation process involved specific cell types: signet ring cells and vanadocytes during the early response, and exclusively vanadocytes after the first 2 days.

Trypan blue was taken up by several types of phagocytic hemocytes after its injection, and subsequently appeared in the epithelial cells of the branchial sac at a time when it had been totally cleared from the blood. Post-phagocytic elimination of india ink through the epithelium of *Ostrea virginica* has been reported by Stauber (1950). Oyster phagocytes were seen to transport the particles through the epithelium of the gut and release them into the external environment. Trypan blue-containing tunicate phagocytes apparently released the dye into branchial sac epithelial cellls, from which it was voided. Unlike the oyster, no dye-laden blood phagocytes were observed within the epithelial cells of the branchial sac. Frequently trypan blue-containing vesicles were seen in almost every cell of the outer portion of the branchial sac. Dye-filled cells were also occasionally seen in the walls of the intestine. Elimination of phagocytosed particles through the digestive epithelia was marked in oyster.

Neither autografts nor allografts were accepted by *M. manhattensis*, probably because they were isolated by mucus production within the surgically prepared pocket in the wall of the recipient's branchial sac. However, a marked hemocyte response to the implanted tissue was observed. This response was characterized by infiltration of the graft by great numbers of vanadocytes. There are resemblances in timing, and in cell types involved, between graft infiltration and encapsulation of glass fragments. In both instances there was an early signet ring cell and vanadocyte response followed by events in which only vanadocytes played a role. It is possible that vanadocytes may function in the ultimate destruction of the graft by rupturing and releasing their strongly acidic contents into the graft. However, their rupture might only reflect necrotic events occurring within the graft.

SUMMARY

Molgula manhattensis was shown to respond to glass fragments and tunicate grafts implanted within the wall of the branchial sac with a number of cellular mechanisms.

Glass fragments inserted into branchial sac tissue were encapsulated by hemocytes. Vanadocytes, or cells derived from vanadocytes, were the blood cells most active in encapsulation mechanisms. Portions of the capsules were composed of: (1.) multilayered structures made up of vanadocytes, (2.) monolayers of cells derived from vanadocyte aggregates, (3.) strands of tunicin produced by vanadocytes.

Injected carmine and trypan blue were ingested by phagocytes of several types. Trypan blue was transferred from the blood phagocytes to epithelial cells of the branchial sac, from which it was eliminated from the animal.

Although acceptance of autografts and allografts was not observed, a marked cellular response to the grafts occurred. This response was characterized by massive infiltration of the graft by vanadocytes.

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DEVELOPMENT, SUBSTRATUM SELECTION, DELAY OF METAMORPHOSIS AND GROWTH IN THE SEASTAR, *MEDIASTER AEQUALIS* STIMPSON

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To understand the functional organization of benthic marine communities, it will be necessary to know more about the nature of recruitment(Thorson, 1957, 1966; Loosanoff, 1964). In general, larvae of benthic animals are selective as to the nature of substratum or micro-habitat in which they set (for examples see Wilson, 1960 and Thorson, 1966), though the degee of specificity may vary with the adult food requirements (Scheltema, 1961). Larvae of certain predators with specific food requirements are known to undergo metamorphosis selectively on the epidermis or other surfaces of the adult's prey (Thompson, 1964). In this report we examine the larval biology of an asteroid which is capable of exploiting a wide range of both foods and habitats, and we examine one habitat which appears favorable for a variety of juvenile asteroids.

Mediaster aequalis Stimpson has the most catholic diet recorded for a seastar (Mauzey, Birkeland and Dayton, 1968). It feeds on plants of at least four phyla, sessile and motile animals of at least nine phyla, detritus and apparently suspended material. *M. aequalis* is found commonly on mud, sand, cobble and rock substrata and is recorded from the low intertidal (unpublished observations) to depths of at least 274 m (Fisher, 1911, page 200). The adults are thus quite broad in their survival requirements and it might be predicted that *M. aequalis* would settle and undergo metamorphosis under a wide variety of conditions. As the larvae are lecithotrophic, possessing no functional mouth nor gut, it might also be predicted that their ability to prolong their larval life when failing to encounter environments suitable for metamorphosis might be severely limited. Neither prediction appears to be true for *M. aequalis*.

OBSERVATIONS AND RESULTS

Spawning

The breeding season of *Mediaster aequalis* in Puget Sound and near the San Juan Islands is probably late March through May. Specimens of *M. aequalis* were seen spawning in the field in late April of 1967 and those kept in running sea water at Friday Harbor Laboratories spawned in late March of 1969. Larvae which appeared to be *M. aequalis* were found in the plankton near Friday Harbor on 30 May 1970. Animals collected in late July 1970 had spent gonads. However, animals held for long periods in aquaria at Friday Harbor spawned as late as July in 1967 and 1968.

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An upper estimate of egg number was calculated for a ripe *M. aequalis* 13 cm in diameter, about the median size of the *M. aequalis* at the field site. The egg volume is about 0.7 mm^3; the gonad volume before spawning about 1.25 ml. If spawning occurs once a year, such an individual contributes less than 1800 eggs annually.

Development

The oocytes are opaque and bright orange and are about 1.0 to 1.2 mm in diameter (Fig. 1). The color persists throughout development and metamorphosis and is similar to that of the adult. The slightly pear-shaped oocytes are buoyant and float at the surface of the water with the large end up. They are surrounded by a layer of striated jelly and the polar bodies appear after shedding.

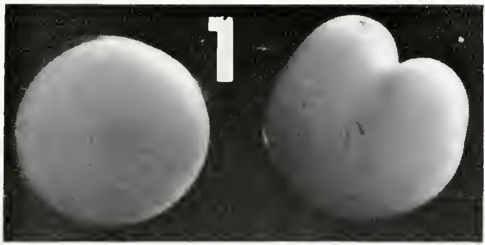
TABLE I

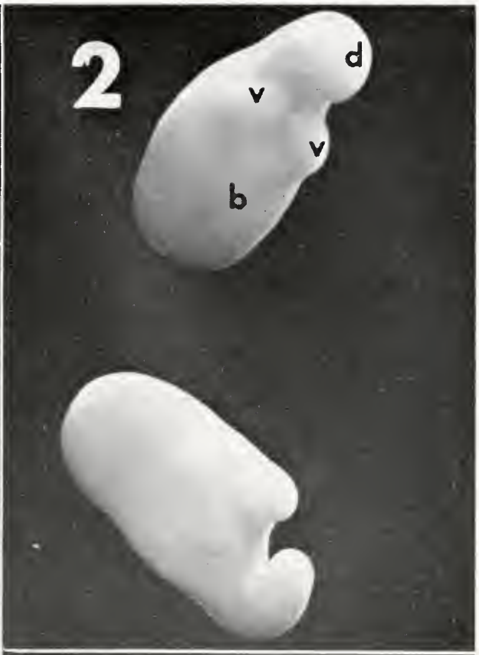
Chronology of normal development of M. aequalis *at 9 to 11° C and with tubes of* Phyllochaetopterus *as a substratum for settling*

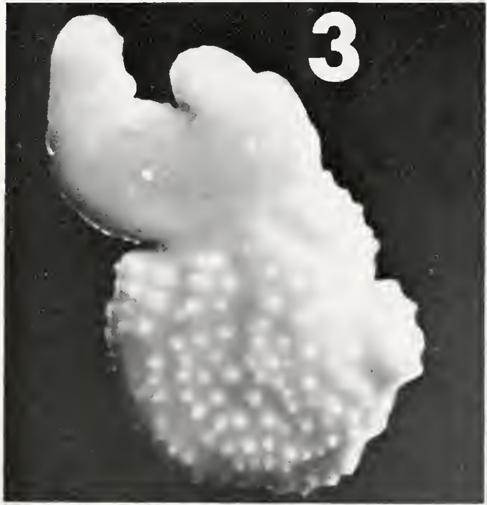
Time	Stage
0 hr	Eggs fertilized
4 hr	1st cleavage
2 days	Morula; early blastula with numerous surface furrows
3 days	Late blastula with few surface furrows
4 days	Gastrula with large blastopore
5 days	Hatched gastrula with small blastopore, surface furrows disappear, elongating along A-V axis
9–10 days	Developing brachiolar arms visible
16 days	Adhesive disk developed
30 days	Larvae fully developed with adult spines visible; larvae have sunk to the bottom and temporarily attached by the brachiolar arms
38 days	Attachment by adhesive disk and completion of metamorphosis by some larvae
14 months	Larvae still capable of metamorphosis

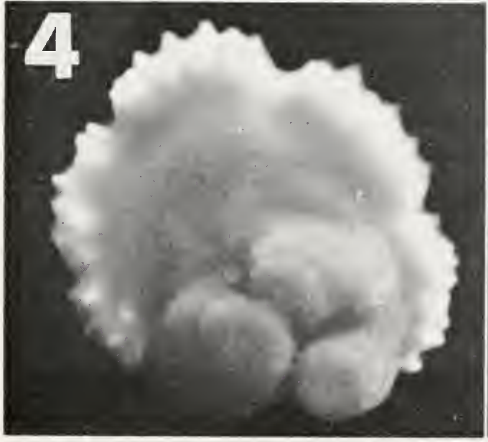
The fertilization membrane is low. Cleavage appears to be holoblastic and seems to follow the typical pattern of radial cleavage in echinoderms. The chronology of development is given in Table 1.

The surface of the blastula is furrowed and is probably infolded like that of *Leptasterias hexactis* (Chia, 1968). The number of surface furrows decreases so that eventually the bastula resembles an embryo at the earlier cleavage stages. One of the surface furrows of the blastula becomes recognizable as the blastopore, which is at first large and irregular. The embryos hatch as ciliated, swimming gastrulae which are still buoyant. As the larva elongates to about 1.9 mm, the blastopore becomes small and round, moving to the ventral side about 0.5 mm from the posterior end. The other furrows disappear.









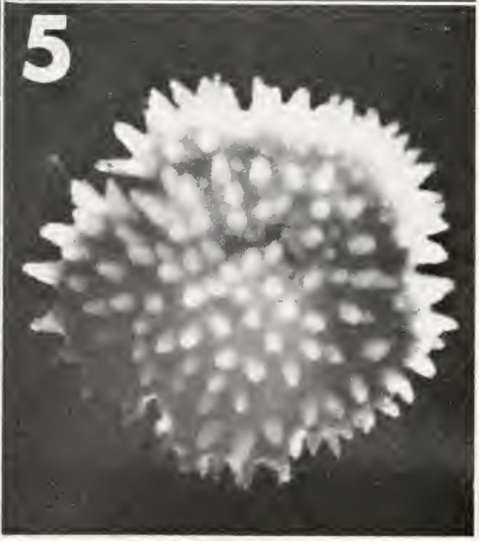


FIGURE 1. Zygote (left); first cleavage beginning (right).

FIGURE 2. Young brachiolaria larvae showing the preoral lobe with three brachiolar arms, one median antero-dorsal (d) and two ventro-lateral (v), and the larval body (b).

FIGURE 3. Advanced brachiolaria larva. Adult spines and rudiments of rays are seen on the larval body.

FIGURE 4. Oral view of metamorphosing juvenile. The preoral lobe is being absorbed (bottom). The mouth has not yet opened.

FIGURE 5. Aboral view of a post-metamorphosis juvenile with extended tube foot visible at upper left.

The larva is lecithotrophic and entirely lacking in feeding structure or mouth, but can be considered a modified brachiolaria. Ten days after fertilization three brachiolar arms, one median anterodorsal and two ventrolateral, begin to form. After 16 days the brachiolar arms are able to adhere to glass needles and pipets and the adhesive disk is visible (Fig. 2). As the brachiolar arms and adult structures develop, the larvae become less buoyant and spend more time at the bottom of the culture dish. Eventually swimming becomes greatly reduced or stops altogether. The fully developed brachiolaria (Fig. 3) is about 2.2 mm long. The form of the brachiolar arms is quite variable. Most individuals have three distinct arms, but some appear to have four, apparently from a division of the antero-dorsal arm. The disk of the starfish at the posterior end of the larva is about 1.2 to 1.3 mm across. The adult mouth and tube feet do not form until after the larva has permanently attached to the bottom. We could see no further development prior to settling.

Fully developed larvae temporarily attach themselves to the glass dish by their brachiolar arms, but in settling, the final attachment is with the adhesive disk. The preoral lobe, which includes the brachiolar arms and adhesive disk, is absorbed (Fig. 4). Metamorphosis is then considered to be complete. The juvenile, about 1.3 mm in diameter, begins its benthic life with two pairs of tube feet per arm. The five arms are of uneven size and difficult to distinguish in aboral view (Fig. 5).

Choice of substratum and delay of metamorphosis

At 25 to 30 days after fertilization the larvae from the 28 March 1969 spawning were temporarily attaching to the glass by their brachiolar arms and appeared ready to settle. At 29 days after fertilization, four substrata which seemed favorable for settling were provided in separate culture dishes. These were (1) two juvenile *Mediaster aequalis* (2.5 cm diameter); (2) sand from an area where adult *M. aequalis* are found; (3) same as (2) but with two small sea pens *Ptilosarcus gurneyi* Gray (1.0 to 1.5 cm tall); and (4) same as (2) but with tubes of the polychaete *Phyllochaetopterus prolifica* Potts (hereafter referred to as *Phyllochaetopterus*). *Ptilosarcus gurneyi* was chosen because it makes up a major portion of the diet of adult *M. aequalis* in the field. *Phyllochaetopterus* tubes were chosen because juvenile specimens of *M. aequalis* are regularly found on them in the field. We set up 3 separate bowls for each kind of substratum, and 3 clean bowls as controls, for a total of 15 cultures. We then placed 15 brachiolaria larvae into each bowl and maintained them at a temperature of 10–14° C on a water table. The results of this test are given in Table II.

The small specimens of *M. aequalis* ate the larvae. We do not know the cause of mortality in the other bowls. Larvae did not settle in plain glass bowls or in the presence of *Ptilosarcus gurneyi*. Larvae first began to settle in the bowls with tubes of *Phyllochaetopterus*, and at 56 days after fertilization, the specimens of *Phyllochaetopterus* appeared to be about 4 times as effective as the sand in inducing settling. Although the larval development in *M. aequalis* had appeared quite synchronous, the fully developed larvae are quite variable in their readiness to settle. At 56 days only half the larvae had settled on the tubes of *Phyllochaetopterus*.

Table II

Metamorphosis and mortality of Mediaster aequalis *with different substrata or species present. Test begun 29 days after fertilization*

Substratum or species tested	Dish number	38 days after fertilization			56 days after fertilization		
		# larvae	# died	# metamorphosed	# larvae	# died	# metamorphosed
glass only	1	15	0	0	15	0	0
	2	12	3	0	12	3	0
	3	15	0	0	14	1	0
juvenile *Mediaster*	1	0	15	0	0	15	0
	2	1	14	0	0	15	
	3	2	13	0	1	14	0
sand	1	15	0	0	10	3	2
	2	15	0	0	14	0	1
	3	14	1	0	9	4	2
Ptilosarcus and sand	1	13	2	0	11	4	0
	2	15	0	0	9	6	0
	3	15	0	0	13	2	0
Phyllochaetopterus and sand	1	12	1	2	8	3	4
	2	12	1	2	6	1	8
	3	12	0	3	6	2	7

Tubes of *Phyllochaetopterus* were more effective in inducing settling and metamorphosis in older larvae. When the larvae were 128 days old, 32 of the 33 larvae that were offered *Phyllochaetopterus* tubes settled and metamorphosed within 9 days. None of the 33 control larvae in a plain glass bowl had metamorphosed. By this age a few larvae had settled and metamorphosed in those culture dishes in which algae had been allowed to grow on the glass. Most larvae did not metamorphose until a more favorable substratum (*Phyllochaetopterus*) was available.

Small plants and animals grow on the tubes of *Phyllochaetopterus* and we made no attempt to determine whether the attraction lay in the tubes themselves or the associated organisms.

Six larvae from the 28 March 1969 spawning were still alive after 14 months. They lay on the bottom of the glass bowl, no longer swimming. Deep divisions of the brachiolar arms gave the appearance of a preoral lobe with 6 to 8 brachiolar arms. The spines on the larval body were well developed. The bright orange larvae now appeared translucent. On 23 May 1970 all six were placed in a bowl with *Phyllochaetopterus*. None had undergone metamorphosis 18 days later, but all had undergone metamorphosis after 51 days.

Growth

Juvenile *Mediaster* were collected from beds of *Phyllochaetopterus prolifica* (at a depth of 20 m MLLW) during four dives in fall and winter, 1968–1969.

The mean and range of size of the smallest 10% of those measured are given in Table III. Changes in mean size at the lower end of the size range could result from settling of larvae during this period, differential mortality, or growth. It seems unlikely that many larvae would be settling more than 5 months after spawning. If we attribute increased size to growth alone, then we find growth rates up to 2 mm/month between November and January or 0.5 mm/month over the whole period from September to March. The increases between November and January and between September and March are significant, but so is the unexplained decrease between January and March ($P < 0.01$, 1-way ANOVA). Juveniles reared at 10–14° C in the laboratory with running sea water, sand, and either small *Ptilosarcus gurneyi* or *Phyllochaetopterus* tubes grew only about 0.3 to 0.4 mm/month, attaining diameters of only 2.7 to 3.7 mm at 6 months after metamorphosis (May to November).

One hundred specimens of *Mediaster aequalis* were tagged by hypodermic insertion of individually numbered FD-67 Floy Tags. These are numbered plastic tubes with an internal cross-bar anchor. Only 3 were recovered after periods of greater than 6 months (Table IV). The absolute rate of increase in mean diameter of adults (0.7 to 1.1 mm per month) does not appear to be much greater than that of the juveniles. These estimates of juvenile and adult growth rates are similar to the lower estimates reported for other species of asteroids (Kenny, 1969; Swan, 1966). Some observations (Feder and Christensen, 1966) indicate slower growth in the colder part of the year, when our field estimates of juvenile growth were made.

TABLE III

Size of the smallest 10% of the Mediaster aequalis *found on* Phyllochaetopterus prolifica *in the field*

Date	Size range (dia. in mm)	Mean size (dia. in mm)	No. in 10% of the sample
13 Sept. 1968	2 to 7	4.2	22
24 Nov. 1968	4 to 7	5.8	9
20 Jan. 1969	7 to 12	9.6	11
25 March 1969	4 to 10	7.4	13

Field observations on juvenile asteroids

The tubes of the polychaete *Phyllochaetopterus* may play a significant role as an "asteroid nursery" in Puget Sound. Recently metamorphosed asteroids (less than 10 mm in diameter) can be found regularly in *Phyllochaetopterus* beds where small *Mediaster aequalis*, *Crossaster papposus* (Linnaeus), *Luidia foliolata* Grube, *Pteraster tesselatus* Fisher, *Henricia leviuscula* Stimpson, *Solaster stimpsoni* Verrill, *S. dawsoni* Verrill and unidentified forcipulates are all common. Small asteroids of all species are often found crawling along the worm tubes with their stomachs everted. They are rarely found on the sand beneath the worm tubes. *Crossaster* (3 and 4 mm in diameter) and *Mediaster* (2 to 4 mm in diameter) have been found on ectoprocts, *Bugula* sp., their stomachs inserted into individual zooecia. Small *Mediaster* and *Pteraster* have been found with their stomachs everted onto the surface of sponges which were growing attached to the *Phyllochaetopterus* tubes. Thus *Phyllochaetopterus* seems to harbor food for very small asteroids of generalized diet. In sand-bottom habitats, tubes of these worms are the only abundant attachment sites for ectoprocts, sponges, colonial and solitary ascidians, brachiopods, hydroids and so on; these animals do not grow to large size on the tubes. Asteroids less than 10 mm in diameter must have a difficult time capturing food; sponges and ectoproct zooids would seem to be suitable prey. Most of the asteroids, however, seem to be feeding generally on microscopic growth of detritus coating the surfaces of the worm tubes. Fifty-one of sixty-four specimens of *M. aequalis* were found with their stomachs everted on the *Phyllochaetopterus* tubes.

Small specimens of *Solaster dawsoni* are found in *Phyllochaetopterus* where they prey upon small asteroids and holothrurians. A 4.8 cm *S. dawsoni*, for example, was seen successfully capturing a 4.9 cm *S. stimpsoni*.

TABLE IV

Growth of adult Mediaster aequalis *in the field*

Total diameter in mm		Time interval in months	Growth (mm diameter)/ month
Beginning	End		
112	123	11.7	0.9
132	138	9.0	0.7
134	141	6.5	1.1

Small (9 and 11 mm) specimens of *Mediaster* on the sand below the worm tubes were, on two occasions, eating ostracods, their stomachs inserted between the valves. Very small bivalves regularly fall prey to slightly larger *Mediaster* and *Crossaster* (10 to 20 mm).

DISCUSSION

Although adult *Mediaster aequalis* occur in many habitats and eat a variety of foods, their larvae are quite selective as to sites for metamorphosis. *M. aequalis* will not settle on clean glass and can postpone metamorphosis for over a year, if suitable substrata are not available. The pelagic larvae of many benthic marine invertebrates delay settling and metamorphosis in the absence of a suitable substratum, becoming less specific in their requirements as time progresses (Thorson, 1966). *Mediaster aequalis* demonstrates the extraordinary length to which such delay of metamorphosis can be carried. Other lecithotrophic, pelagic asteroid larvae have not delayed so long in the laboratory (see, for example, Chia, 1966; Gemmill, 1912, 1920; Kempf, 1966; Mortensen, 1938).

We were surprised that the larvae could survive 14 months and still complete metamorphosis. The larvae cannot feed during this time. If the larva subsists entirely on energy reserves from the egg, the metabolic rate must be quite low.

The larvae might take up dissolved organic matter, as has been reported for adult starfish (Ferguson, 1967), polychaete larvae (Bass, Chapman and Chapman, 1969), and brooded ophiuroid larvae (Fontaine and Chia, 1968), but at least some small organisms excrete more organic matter than they take up (Johannes, Coward and Webb, 1969), so there may be no net gain.

Since the larvae of *M. aequalis* can survive 14 months, they might be termed "long-distance larvae" in the sense of Thorson (1961). However, these larvae probably could not remain pelagic for this long under natural conditions. During the period of delay they tend to sink. Once near the bottom they would probably come in contact with a favorable substratum or be eaten within a fairly short time.

At the location of this study, juvenile *Mediaster aequalis*, and also juvenile *Luidia foliolata*, *Crossaster papposus*, *Henricia leviuscula*, *Solaster stimpsoni*, *S. dawsoni* and *Pteraster tesselatus* were commonly found on tubes of the polychaete *Phyllochaetopterus prolifica* and were rarely found elsewhere. *Phyllochaetopterus* tubes were more effective in inducing settling by *Mediaster* than the other substrata tested. It is therefore tempting to speculate that the beds of *Phyllochaetopterus* tubes, with the associated small organisms, are a favorable "nursery ground" for juvenile starfish, providing an attractive site for settling and an abundance of food in the form of epizoites. After a few years the starfish presumably would move out to the sandy areas and eat larger prey. Our data are consistent with this view but do not prove it correct. Other substrata in nature may be more attractive, or just as attractive, as the tubes of *Phyllochaetopterus prolifica*.

We do not know the extent to which juvenile *M. aequalis* are preyed upon in the beds of *Phyllochaetopterus*. Small *Solaster dawsoni*, a major predator of asteroids, are particularly abundant on *Phyllochaetopterus* tubes, so safety from predation may not be a feature of this habitat. Laboratory observations of the cannibalism by the juveniles on settling larvae suggests a mechanism by which further recruitment might be limited after a heavy set. The observation of *M. aequalis* up to 35 mm in diameter, on *Phyllochaetopterus* suggests that *M. aequalis* may remain on *Phyllochaetopterus* for 2 or 3 years, patrolling the tubes on which the branchiolariae of their species set. A particularly abundant recruitment of *Mediaster* in one year could impose higher mortality on the next two year classes.

The evidence available suggests a very slow growth rate for *M. aequalis*. If *M. aequalis* increases in diameter at a rate no greater than 2 mm per month, then at least 4 years would be required to reach sexual maturity. A median-size adult produces less than 1800 eggs in a single spawning and probably spawns only once a year. The larvae spend at least 4 weeks exposed to the dangers of a planktonic existence. Settling in a favorable habitat and the characteristic of the juvenile formed at metamorphosis are obviously of critical importance to the perpetuation of this abundant species.

Hyman (1955, page 305) remarks that "The baby stars are of microscopic dimensions, less than 1 mm across when developing from small nonyolky types of eggs, 1 to 2 mm across when coming from large yolky eggs." A review of the literature indicates that this remark also applies to planktotrophic development as compared to lecithotrophic development in asteroids. This is confirmed by our experience in regions near Puget Sound where *Pteraster tesselatus, Mediaster aequalis, Solaster stimpsoni* and *Hippasteria spinosa* Verrill with pelagic lecithotrophic larvae form juveniles greater than 1 mm, and *Pisaster ochraceus* (Brandt), *Pycnopodia helianthoides* (Brandt), *Luidia foliolata* and *Patiria miniata* Brandt with pelagic planktotrophic development form juveniles closer to 0.5 or 0.6 mm diameter (Chia, 1966; Greer, 1962; and unpublished observations). A notable exception to this trend in asteroids are the planktotrophic larvae of some species of *Luidia* which produce very large juveniles (Tattersall and Sheppard, 1934). The production of large juveniles by lecithotrophic larvae must necessitate more organic material per egg and therefore fewer eggs than would otherwise be possible. The production of larger juveniles by planktotrophic development probably would require a longer period in the plankton. Presumably larger juveniles have an advantage in obtaining food or avoiding predation. If these assumptions are correct, then the advantage gained by producing a larger juvenile generally exceeds the cost of fewer eggs in asteroids with lecithotrophic development, but is generally less than the cost of a longer period in the plankton in asteroids with planktotrophic development.

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SUMMARY

1. The eggs of *Mediaster aequalis* (Asteroidea, family Goniasteridae) are 1.0 to 1.2 mm in diameter. Development is lecithotrophic with a wrinkled blastula and modified brachiolaria larva. The metamorphosed juveniles are about 1.3 mm diameter. Estimated growth rates are less than 2 mm per month.

2. In the laboratory, *M. aequalis* larvae settled on tubes of the polychaete *Phyllochaetopterus prolifica*, but not on other substrata tested, 38 days after fertilization.

3. If *Phyllochaetopterus* tubes were not present, metamorphosis was postponed. Some larvae survived 14 months and still completed metamorphosis when offered tubes of *Phyllochaetopterus*.

4. In the laboratory, juvenile *M. aequalis* ate settling *M. aequalis* brachiolaria larvae.

5. Juvenile *M. aequalis* and juveniles of several other asteroid species were found commonly on tubes of *Phyllochaetopterus*. Their feeding was observed in the field. Tubes of *Phyllochaetopterus* may play a significant role in the life of asteroids in Puget Sound.

6. In asteroids, lecithotrophic development is generally associated with formation of larger juveniles at metamorphosis (Hyman, 1955). Possible implications of this phenomenon are discussed.

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RESPIRATORY ADAPTATIONS TO THE OXYGEN MINIMUM LAYER IN THE BATHYPELAGIC MYSID *GNATHOPHAUSIA INGENS*

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Zones of minimum oxygen are found at intermediate depths in most of the world's oceans and, although the dissolved oxygen in some of these "oxygen minimum layers" is considerably less than 0.5 ml/l, populations of metazoans exist there (Schmidt, 1925; Sewell and Fage, 1948; and Banse, 1964). Previous studies have shown that two crustaceans which live in such minimum layers are unusually effective at removing oxygen from water (Teal and Carey, 1967; Childress, 1968a). One of these organisms, the lophogastrid mysid *Gnathophausia ingens,* is apparently capable of living largely aerobically at oxygen concentrations as low as 0.20 ml O_2/l (Childress, 1968a). The ability to regulate oxygen consumption down to this low level is shared with other inhabitants of the oxygen minimum layer (Childress, 1968b and 1969) and is unique among previously studied metazoans.

The relationship between crustacean respiratory adaptations and physiological mechanisms such as ventilation rates and utilization of available oxygen have been investigated only in a few species of crustaceans inhabiting regions with abundant oxygen (Thomas, 1954; Larimer and Gold, 1961; Arudpragassam and Naylor, 1964; Moshiri, Goldman, Godshalk and Mull, 1970). This report examines the adaptive mechanisms that allow *G. ingens* to be so effective in the regulation of its oxygen consumption rate at low oxygen concentrations.

METHODS AND MATERIALS

Animals

Most of the specimens of *Gnathophausia ingens* (Dohrn, 1870) used were taken in the basins off southern California with a ten foot Isaacs-Kid midwater trawl from the Research Vessel VELERO IV. However, some of the specimens used were captured in the same region with a six-foot-square Tucker trawl from the Research Vessel TE VEGA. Immediately after they reached the surface, the animals were placed in one gallon polyethylene jars which were full of cold (3° C) seawater. The animals were then transported to either Hopkins Marine Station or the Santa Barbara Marine Laboratory where they were maintained in seawater at 5 to 7.5° C. Individuals of *G. ingens* have now been maintained for more than a year on a diet of salmon, shrimp and bonito. All of the experimental animals were sexually immature individuals of undetermined sex and had a wet weight less than 13 g.

Respiration measurements

Oxygen consumption rates at different oxygen concentrations were determined for individuals sealed in a chamber filled with seawater and maintained at 5.5 ± 0.1° C by means of a refrigerated water bath. The inner part of the chamber to which the animal was exposed was constructed of pyrex with a lucite lid. The rate of change in oxygen partial pressure in the chamber was continuously measured with a Clark-type oxygen electrode (Clark, 1956) as the animal reduced the oxygen partial pressure from air saturation to unmeasurably low partial pressures (less than about 0.1 mm Hg). The time required for this reduction ranged from 12 to 32 hours. The electrode tip was enclosed in a perforated plastic vial containing a magnetic stirring bar which stirred the electrode and the contents of the chamber. To avoid excitation of experimental animals by light, the experimental chamber was kept in darkness during each experiment. To control pH, I used seawater buffered with 2.5 g per liter of tris(hydroxymethyl)amino-methane adjusted to pH 8.0 with HCl, and diluted to the appropriate osmolality with distilled water. To test the effect of waste product accumulation on the respiration of experimental animals, the chamber was filled with water saturated with 95 per cent oxygen and 5 per cent carbon dioxide. Since there was more oxygen in the chamber, the animals could be maintained in the chamber about five times longer than usual. This should have resulted in a build-up of far more waste products during the course of the experiment. Yet the data obtained were indistinguishable from those obtained by the usual method. Therefore it was concluded that waste product build-up did not affect the results.

Streptomycin (6 mg/l) and aureomycin (20 mg/l) were added to the seawater to minimize microbial growth. As a control on microbial respiration, the animal was removed from the chamber at the end of each experiment, air-saturated seawater was added to replace the volume of the animal, and the rate of oxygen consumption in the chamber was again measured for 3 to 24 hours. These rates, which were independent of oxygen concentration, constant with respect to time and always less than 5 per cent of the total measured rate, were subtracted from the respiratory rates measured with the animal in the chamber, to obtain the respiratory rates of each animal. The rate of maximum activity was measured by using a chamber without the plastic sieve that shielded the animal from the stirring bar. The bar was then operated at the highest speed that still allowed the animal to maintain its position in the chamber.

The oxygen electrodes were polarized at 0.7 v by means of a voltage divider and an "alkaline" type battery, and the resulting potential difference across a 20 kohm potentiometer was continuously recorded on potentiometric stripchart recorders. The electrodes were calibrated before and after each run with air-saturated and nitrogen-saturated seawater (99.99%) at the experimental temperature. If the initial and terminal air calibrations differed by more than 2%, or if the nitrogen calibrations differed measurably, runs were discarded. Rates of oxygen consumption were calculated using the oxygen solubility tables of Green and Carritt (1967). Because the animals were excited by handling, the recordings during the first 5 to 6 hours were disregarded.

Since oxygen electrodes are very sensitive to stirring, it was possible to measure the swimming activity of an animal by fastening the animal in one position and placing an oxygen electrode near the animal's pleopods. The activity and respiratory rates of animals were monitored simultaneously in several runs by fastening the animal in the above manner in a respiration chamber containing a second, isolated electrode agitated by a stirring bar. The animal's activity was indicated by the difference in readings between the animal-stirred electrode and the electrode stirred at a constant rate by the magnetic stirrer.

Respiratory rate, oxygen in exhaled water, and flow rate of water over the gills were measured simultaneously in the following manner. The animal's head was placed in a cylindrical plastic vial and a piece of rubber balloon sealed the animal to the vial. Water was drawn in under the carapace, passed through the gills and then pumped out of the carapace into the vial where it first passed a Beckman Macro Oxygen Electrode and then a thermistor flowmeter. The term ventilation volume will be used to refer to the rate of volumetric flow of seawater over the gills. The flowmeter was a thermistor probe (Yellow Springs Instrument Co. model 403) with 30 inches of 30-gauge nichrome wire wrapped around the tip and insulated with epoxy enamel. This wire was heated with 400 ma at 3 volts and the resulting thermistor temperature was proportional to the flow rate past it. The flowmeter was calibrated by measuring the amount of water that had run past it in a given period of time. The precision of the flowmeter was about ±10% between 0.5

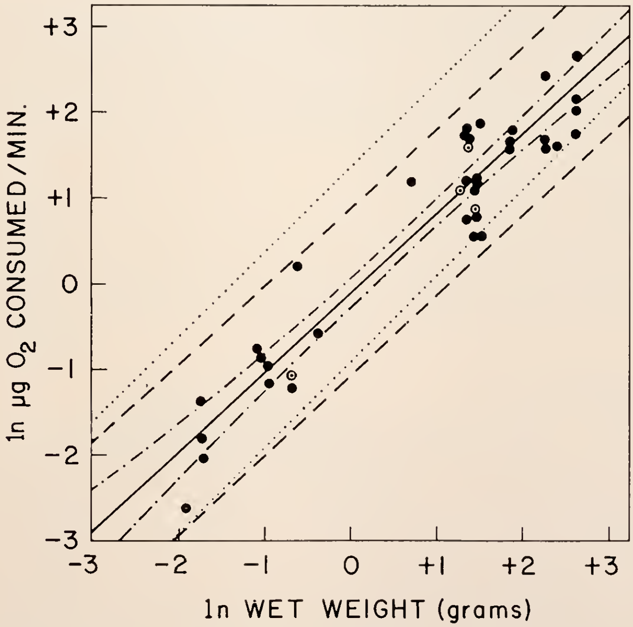

FIGURE 1. Relation between (ln) size and (ln) respiration (lowest sustained rates, see text) in *Gnathophausia ingens* at 5.5° C; ——— regression line ln $R = -0.105 + 0.925$ ln W, $R = 0.900\ W^{0.925 \pm 0.10}$, – · – – · – – · – 95% confidence interval for mean R at given W, – – – – – – – – – 95% confidence interval for individual R at given W, ... envelope around all measured respiration rates; for upper line respiration equals 3.6 mg/kg/min, for lower line respiration equals 0.4 mg/kg/min, ⊙ means that two data points are at that locus, • means that a single datum point is at that locus.

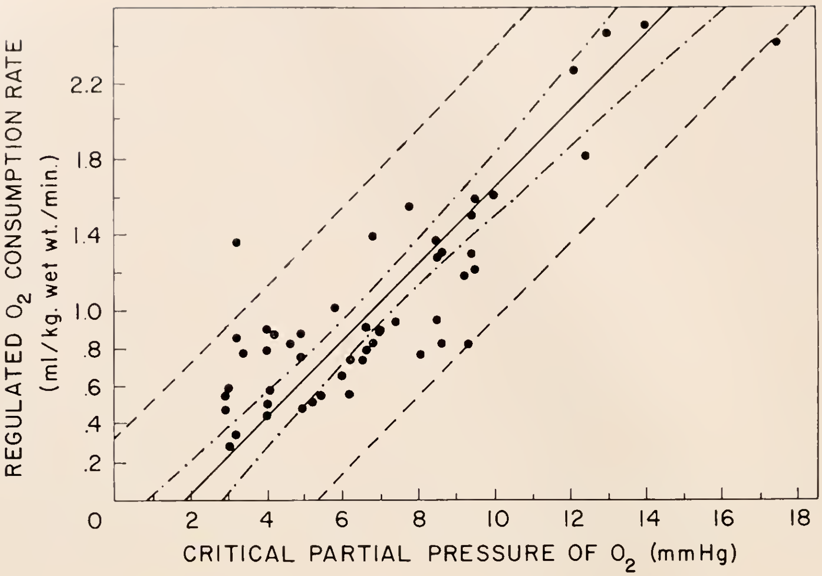

FIGURE 2. Relationship between respiration and P_c in *Gnathophausia ingens*; ———— regression line $X = 4.973Y + 1.798$ (X as dependent variable) 95% confidence 4.973 ± 0.889, 1.798 ± 0.470; $r = 0.85$, -·--·--·- 95% confidence interval for mean X at a given Y, ---------- 95% confidence interval for individual X at a given Y. Regulated oxygen consumption rate is defined as the lowest rate sustained for more than 10 minutes between 20 and 70 mm Hg of oxygen. Critical partial pressure (P_c) is defined as the partial pressure of oxygen at the point where the lowest sustained rate intersects the dependent part of the curve or its extension.

and 50 ml/minute. Flow rate was also calculated from the measured oxygen consumption rate and the amount of oxygen removed as the water passed over the animal's gills. A Beckman Macro Electrode was used to monitor oxygen in exhaled water because this electrode is insensitive to variations in flow rate.

RESULTS

Oxygen consumption rate

The respiratory rates measured during a single run varied a great deal, probably due to "spontaneous" variations in the experimental animal's activity. This variation made it extremely difficult to assign a single representative value for the respiratory rate of an animal for the duration of a given experiment. *Gnathophausia ingens* is a very active and readily excited animal, and the lowest activity which it usually shows in the laboratory is slow swimming. For this reason, I have used the lowest sustained (*i.e.*, sustained for at least 10 minutes) rate between the oxygen partial pressures of 20 and 70 mm Hg as the "assigned" respiratory rate for a given run. These rates were plotted against the wet weight of the animal and the regression equation of oxygen consumption as a function of wet weight was fitted by the least squares method (Fig. 1). The equation determined for 42 experiments with 26 animals was $R = 0.90\ W^{0.93 \pm 0.10}$ where $R = \mu g\ O_2$/min and W = wet weight in grams.

The exponent of the weight was not significantly different from 1.0, the exponent for weight specific respiration ($P < 0.05$). Consequently, all subsequent data are presented on a wet weight specific basis.

In those runs where respiration and activity were measured simultaneously the respiratory rate of nonswimming animals was between 0.40 and 0.45 mg O_2/kg wet wt/minute. The "active" rate, determined for 5 animals, was between

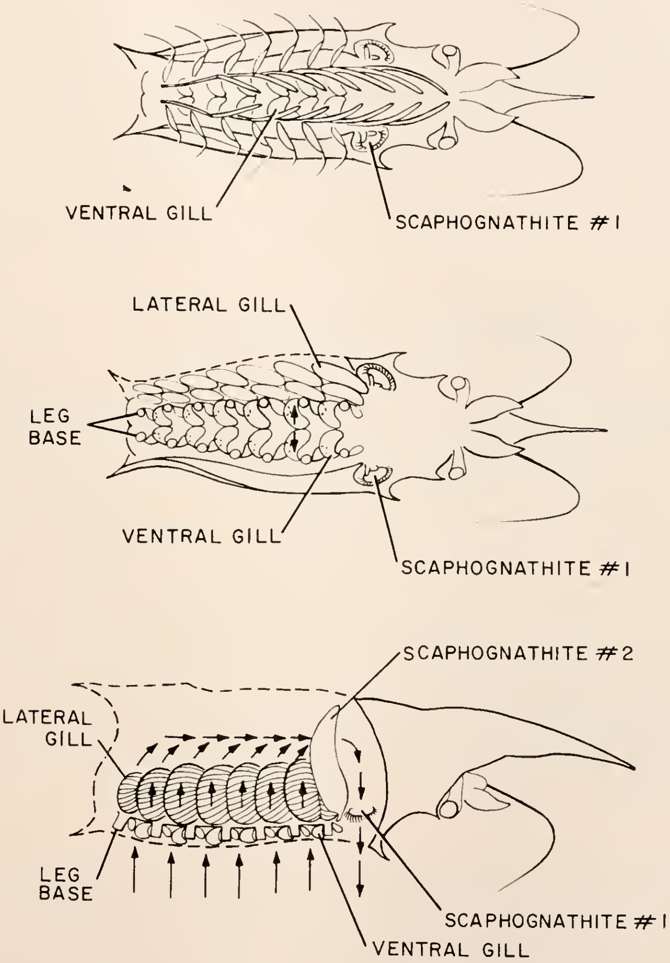
VENTRAL GILL
SCAPHOGNATHITE #1
LATERAL GILL
LEG BASE
VENTRAL GILL
SCAPHOGNATHITE #1
SCAPHOGNATHITE #2
LATERAL GILL
LEG BASE
SCAPHOGNATHITE #1
VENTRAL GILL

FIGURE 3. (A.) Ventral view of the cephalothorax of *Gnathophausia ingens*. Water is drawn between the legs midventrally into the ventral gills. Scaphognathite 1 is the expodite of the second maxilla. Scaphognathite 2 is the epipodite of the first trunk limb. (B.) Ventral view of the cephalothorax of *G. ingens* with legs and one side of the carapace removed (schematic representation not absolutely accurate). The respiratory water passes laterad through the ventral gills, then turns dorsad and flows through the lateral gills. (C.) Side view of the cephalothorax with the carapace removed (position indicated by dashed line). The respiratory water (indicated by arrows) passes in a dorsal direction through the lateral gills. It is then pumped forward, down and out of the carapace by the two scaphognathites.

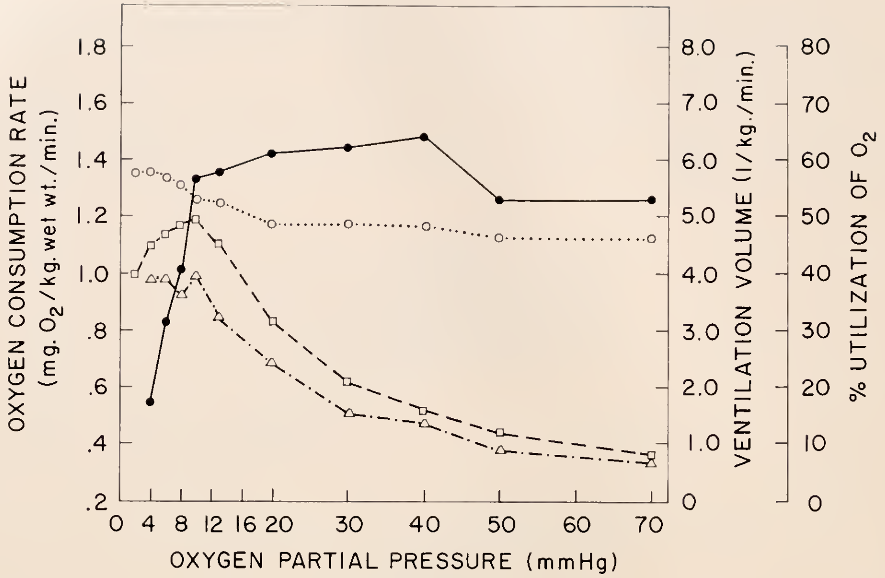

FIGURE 4. Oxygen consumption rate, per cent utilization of oxygen, and ventilation volume in *Gnathophausia ingens* as functions of oxygen partial pressure, mean of 8 runs; ——— oxygen consumption rate, ... % utilization =

$$\frac{\text{PO}_2 \text{ of inhaled water} - \text{PO}_2 \text{ of exhaled water}}{\text{PO}_2 \text{ of inhaled water}} \times 100\%$$

- - - - - - - - measured ventilation volume, —·—·—·— calculated ventilation volume =

$$\frac{\text{O}_2 \text{ consumed/min}}{\text{O}_2 \text{ removed/1 water passed over gills}}.$$

2.6 and 3.6 mg O_2/kg wet wt/minute. Visual observations of individuals in the respiration chamber suggested that a respiratory rate of about 0.8–0.9 mg O_2/kg wt wt/minute (approximately the same as the rate expressed in the regression equation) corresponded to a slow rate of swimming just sufficient to allow the animal to maintain its position in the water column.

Regulation of oxygen uptake

The ability of *G. ingens* to regulate its oxygen uptake was studied in 50 runs with 34 animals of between 0.14 and 13 g wet weight. Although *G. ingens* has been shown to regulate its respiratory rate to a very high degree (Childress, 1968a), a single critical partial pressure (P_c) below which the species does not regulate cannot be defined because the P_c varies with the respiratory rate and the respiratory rate is quite variable (due to activity variations). This relationship is shown in Figure 2. Experimental animals continued to consume oxygen at partial pressures below the P_c until no oxygen was detectable in the chamber; but they continued swimming for less than 30 minutes after they had exhausted the supply of oxygen. These animals could be revived with aerated water up to 6 hours after their supply of oxygen had been exhausted.

Path of respiratory water flow

The resipratory currents of *G. ingens* were traced by placing water containing dyes at different points around the carapace of living animals and observing the path taken by the dye. Most of the respiratory water entered between the left and right sets of legs into the ventral gills (Fig. 3); it passed laterally through the ventral gills and into the lateral gills. The water then passed dorsally through the lateral gills, flowed anteriorly above them, and then turned downward and passed out through the exhalant openings. No water entered under the posterior dorsal edge of the carapace. A little may enter at the base of the thoracic exopodites. The structures responsible for this water flow are the two pairs of scaphognathites. The anterior scaphognathite on each side is the exopodite of the second maxilla; the posterior one is an epipodite on the first thoracic limb.

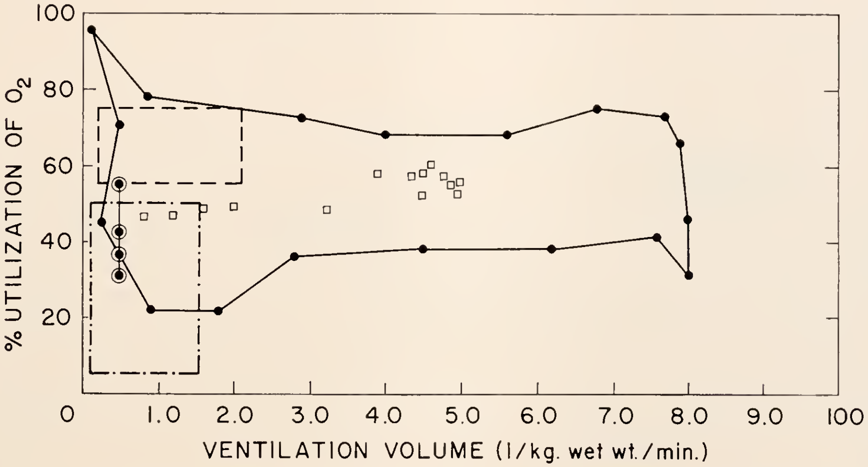

FIGURE 5. Relation between percent utilization and ventilation volume in *Gnathophausia ingens*; ☐ *G. ingens*—mean points taken from Figure 21, •————• *G. ingens*—envelope enclosing all values found, – – – – – – – – – – – *Procambarus simulans*, from Larimer and Gold (1961) – · – · – · *Carcinus maenas*, from Arudpragassom and Naylor (1964), ⊙————⊙ *Homarus vulgaris*, from Thomas (1954).

The illustrations of the gills diagrammatically simplify their very complexly branched and foliaceous structure (Fig. 3). The gill surface area is certainly extremely large; but, because of the great irregularity of the smaller gill divisions, it was not possible to make a satisfactory quantitative estimate of the gill surface area.

Mechanics of oxygen uptake

Ventilation volume, per cent utilization, and oxygen consumption were measured in a series of 8 runs using 6 different individuals between 6 and 8 g wet wt (Fig. 4). Individual *G. ingens* had a low ventilation volume at higher oxygen partial pressures and increased this ventilation volume at lower oxygen levels. The disagreement between the calculated and measured flow rates is probably a result of the combined errors of the two oxygen electrodes and the flowmeter. The highest ventilation volume generally occurred at the P_c, but ventilation remained high until all of the oxygen was consumed. Although the mean curve showed a rather constant per cent utilization, at lower oxygen partial pressures, it actually increased slightly in some cases and decreased slightly in others.

The presentation of these data in Figure 4 masks the short-term changes in the different parameters. Figures 6 and 7 show two sections of recordings on which Figure 4 is based. These recordings show that individuals of *G. ingens* ventilated intermittently at oxygen partial pressures above 15–20 mm Hg and pumped continuously below this level. These recordings also show that, in the short-term, higher ventilation volumes were usually associated with lower per cent utilization.

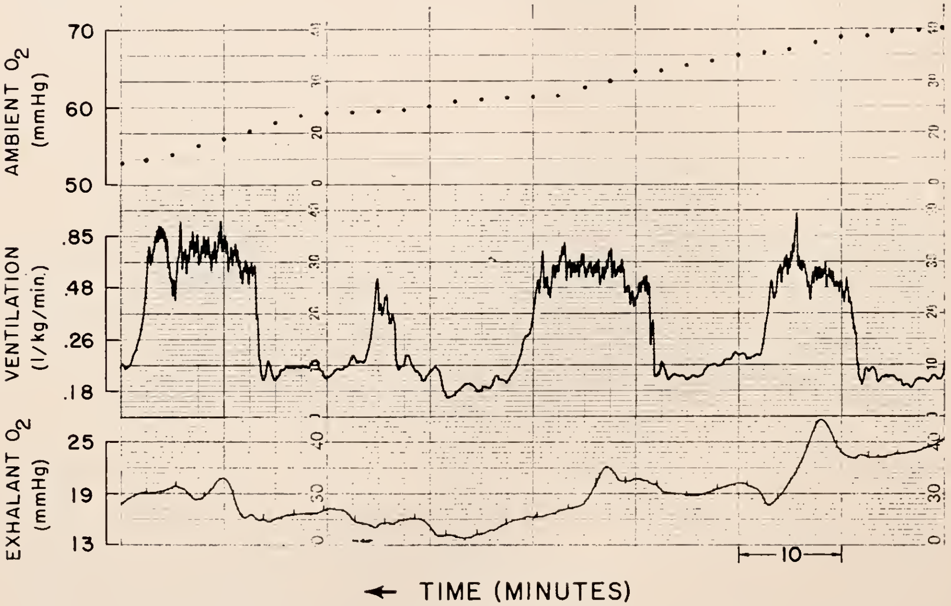

FIGURE 6. Recordings of ventilation volume and exhalant oxygen and an abstraction of the recording of oyxgen in the chamber (Ambient O_2).

DISCUSSION

Most crustaceans previously studied have a respiratory rate which, unlike that of *G. ingens,* is proportional to body surface area (Wolvekamp and Waterman, 1960). However, *Euphausia pacifica,* the other extensively studied pelagic crustacean, has a respiratory rate that is directly proportional to weight (Paranjape, 1967; Small and Hebard, 1967). The respiratory rate of *G. ingens* is clearly not surface proportional, but the 95% confidence interval is broad enough that it is not significantly different from either the intermediate proportionality found for a series of arctic and tropical crustaceans by Scholander, Flagg, Walters and Irving (1953) or from weight proportionality. The significance of weight-proportional (or nearly weight-proportional) respiration in pelagic crustaceans is not clear.

The absolute value of *G. ingens*' respiratory rate is rather low compared to crustaceans previously examined (Wolvekamp and Waterman, 1960; Childress, 1968b). This may well be an adaptation to the paucity of food in the deep-sea (Childress, 1971). The nine-fold range of metabolic rate in *G. ingens* is comparable to that of free-swimming fishes (Brett and Sutherland, 1965) and somewhat higher than is usual for crustaceans. Presumably this wide respiratory range allows the animal to conserve energy by slow swimming most of the time while retaining the capacity for extremely rapid swimming either to capture prey or to escape predators.

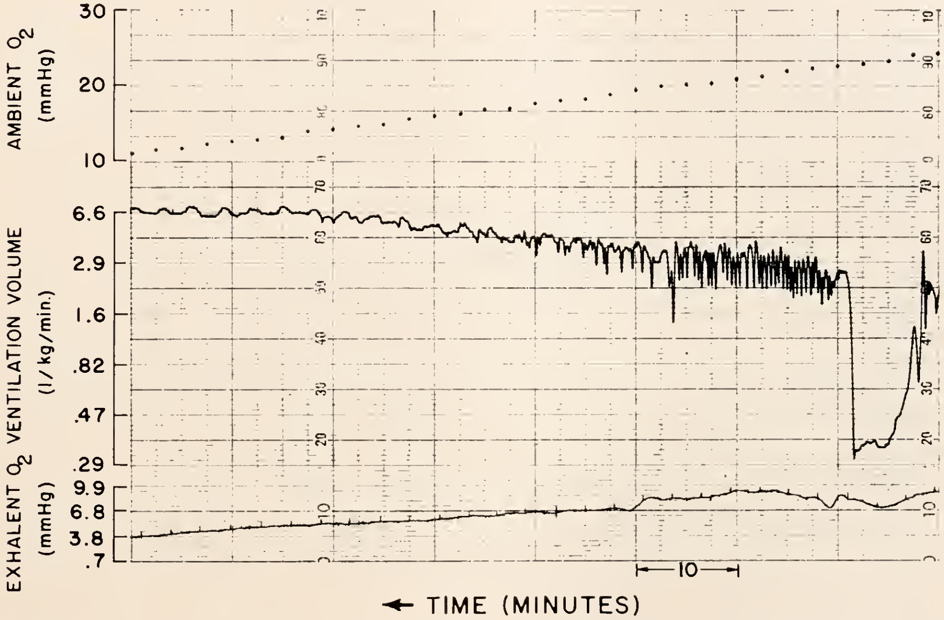

FIGURE 7. Recordings of ventilation volume and exhalant oxygen and an abstraction of the recording of oxygen in the chamber (Ambient O_2). (The pattern seen in the ventilation volume recording at oxygen partial pressures below about 11 mm Hg is not due to the animal but is a result of fluctuations in the heater voltage source.)

The direct proportionality between the P_c and respiratory rate might be expected but it has not previously been demonstrated for invertebrates or fishes. This proportionality offers the possibility of gaining insight into the *in situ* respiratory rate of *G. ingens*. Since this species lives continuously and grows in the oxygen minimum layer off the southern California coast and yet has very little anaerobic capability, it must live largely aerobically at oxygen partial pressures around 6 mm Hg (Childress 1968a, 1968b, 1969 and in preparation). The present data show that at this oxygen level the mean respiratory rate cannot exceed about 0.8 ± 0.1 mg O_2/kg wet weight/min. Visual observations suggest that this respiratory rate corresponds to slow swimming by the animal (approximately the minimum level of swimming activity required for individuals to maintain their position in the water column). It seems likely therefore that individuals of this species when in the minimum layer spend most of their time respiring at about 0.8 mg/kg/min, swimming just rapidly enough to maintain their position in the water column, and relying on anaerobic metabolism for short bursts of greater activity.

Manton (1928) has hypothesized on the basis of preserved specimens of *G. ingens* that the respiratory current flow in this species resembled that in the neritic mysid *Hemimysis lamornae.* She proposed that the main flow of respiratory water enters *G. ingens* (as it does in *Hemimysis* and all other previously studied mysids) under the posterior dorsal edge of the carapace. My observations disagree with Manton's hypothesis showing instead that almost all respiratory water enters the gills in the ventral mid-line and proceeds laterally and dorsally through the gills (Fig. 5). This observed pattern of flow passes all of the respiratory water through the gills and provides a relatively long path-length through the gills. Although I was unable to measure the surface area of the gills because they are very complex and irregular, it is obvious that *G. ingens* has a very large gill surface area compared to crustaceans that live in higher oxygen partial pressures (including *Gnathophausia gracilis, Gnathophausia gigas* and adult females of *G. ingens*). However, the gills of *G. ingens* appear roughly comparable in size to those of caridean and peneid decapods which inhabit the minimum layer. Unusually large gills have also been found in fishes that occur in the oxygen minimum layer (Parin, 1961, Ebeling and Weed, 1963; Gibbs and Hurwitz, 1967). This large surface area is undoubtedly an important factor in explaining the ability of *G. ingens* to extract oxygen so effectively.

The pattern or respiration, ventilation and per cent utilization of oxygen shown in Figure 4 contrasts sharply with that found in studies of crustaceans that inhabit waters high in oxygen (Thomas, 1954; Larimer and Gold, 1961; Arudpragassam and Naylor, 1964, Moshiri *et al.,* 1970). In particular these species use their maximum ventilation volume at or near air saturation and as the oxygen partial pressure is reduced the ventilation volume either decreases or remains constant. The per cent utilization in these species is roughly independent of ventilation volume and either remains constant or increases as the oxygen partial pressure decreases. *G. ingens,* however, holds its per cent utilization relatively constant as the oxygen partial pressure is reduced and regulates its removal of oxygen from the water by greatly increasing its ventilation volume at lower oxygen partial pressures. In addition *G. ingens* can pump far more water over its gills than can any crustaceans previously examined and extract as much or more oxygen from the water in the process (Fig. 5).

The maximum ventilation volumes observed in fishes (Saunders, 1962) correspond roughly to the maximum volumes shown by individuals of *G. ingens.* However, *G. ingens* is vastly superior in regulatory ability to these fishes because ventilation volume and per cent utilization are inversely related in fishes (Fry, 1957; Saunders, 1962; Holeton and Randall, 1967) but independent of one another in *G. ingens* and other crustaceans. Therefore, although at the maximum ventilation volume a fish may be able to pump as much water over its gills as can an individual of *G. ingens*, the individual of *G. ingens* can remove two to six times more oxygen from the water. The inverse relation between ventilation volume and per cent utilization in fishes has been attributed to deformation of gill lamellae away from optimal positions at higher flow rates. It may well be that the characteristic independence of these parameters in crustaceans is due to the greater rigidity (readily observed in gross dissection) of crustacean gills, which are covered with exoskeleton.

Another important difference between the regulatory patterns of fishes and of *G. ingens* is that the P_c of fishes usually corresponds to a precipitous drop in ventilation volume (Beamish, 1964; Holeton and Randall, 1967), while the P_c in *G. ingens* corresponds to a plateau or slight decline in the ventilation volume. This pattern suggests that the failure of respiratory regulation in fishes is the result of the failure of some internal mechanism to function at low internal oxygen levels. The failure of *G. ingens* to regulate below its P_c, however, may simply result from reaching the animal's maximum sustainable ventilation volume. This combined with the relatively constant per cent utilization causes a decrease in respiratory rate proportional to the decrease in oxygen concentration (dependent respiration) below the P_c.

The remarkable adaptation of *G. ingens* to living at low oxygen partial pressures is strikingly indicated by the fact that individuals need not ventilate continuously until the environmental oxygen partial pressure falls to less than 20 mm Hg.

That extreme aerobic adaptations do not characterize animals of the intertidal mud-flats, which often encounter anoxic conditions, or other neritic or fresh water species seems surprising at first. However, these extreme adaptations are probably peculiar to residents of the oxygen minimum layer because its stability has allowed its residents to evolve aerobic adaptations to survive in a very low oxygen environment and thereby take advantage of the energy bonus of aerobic as opposed to anaerobic respiration. On the other hand, oxygen partial pressures in other low oxygen habitats fluctuate widely, often rapidly giving way to total anoxia. Therefore residents of these other low-oxygen habitats either breath air or endure temporary anaerobiosis but do not effectively extract oxygen at low partial pressures. Because oxygen partial pressure in the oxygen minimum remains constantly low, its residents can not repay a considerable oxygen debt, and therefore they lack adaptations allowing for extended periods of anaerobiosis. A possible exception might be a resident, such as a parasite, with a superabundant food supply. Non-resident, "visiting" species in the oxygen minimum which include diel vertical migrators or resting stages (Longhurst, 1967) may be adapted anaerobically. Such adaptations would impose on them no requirement for extra energy because anaerobic end-products could be metabolized during the period of time that they spend out of the oxygen minimum layer.

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SUMMARY

1. The oxygen consumption rate in *G. ingens* is not surface proportional, but is not significantly different from weight proportionality or intermediate proportionality.

2. The critical partial pressure of oxygen in *G. ingens* is directly proportional to the regulated oxygen consumption rate. This relationship suggests that a respiratory rate of about 0.8 ml O_2/kg wet wt/min (corresponding to a slow rate of swimming probably just sufficient to allow an individual to maintain its depth in the water column) is the maximum which *G. ingens* can sustain over long periods *in situ* in the minimum layer.

3. Respiratory water enters the gills along the ventral midline, travels through them first laterally and then dorsally, finally exiting past the two scaphognathites located anterior to the gills. No respiratory water enters at the posterior dorsal margin of the carapace. The large gill surface of *G. ingens* is almost certainly an important factor in its unusual aerobic abilities.

4. The ability of *G. ingens* to maintain a high ventilation volume and a high per cent utilization independent of each other is certainly important in explaining its aerobic abilities. The regulatory abilities of *G. ingens* are probably limited by its maximum ventilatory abilities.

5. It is suggested that the extreme stability of the deep sea made possible the evolution of the specialized aerobic adaptations found in *G. ingens*. This situation contrasts sharply with that of inhabitants of less stable low oxygen environments as well as "commuters" in stable low oxygen environments which can temporarily use anaerobic metabolism without an energy penalty and which would therefore gain negligible selective advantage from specialized aerobic adaptations.

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UPTAKE AND RELEASE OF FREE AMINO ACIDS BY STARFISHES[1]

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In the last few years a considerable quantity of data has been accumulated to indicate that many species of marine invertebrates possess the ability to remove dissolved amino acids and other nutritional compounds directly from sea water, even when these materials are present in very low concentrations. This phenomenon was first clearly demonstrated by Stephens and Schinske in 1961 on representatives from 10 phyla, including Echinodermata. Stephens and his colleagues have continued to examine several aspects of the uptake of organic compounds by various marine species, including a coral, sipunculid, annelids, and brittlestars. Their observations are presented in a series of reports (Stephens, 1962, 1963, 1964; Stephens and Virkar, 1966; Virkar, 1966).

My own work (Ferguson, 1963, 1967a, 1967b, 1968a; 1969, 1970) has confirmed the ability of several starfishes to take up dissolved organic compounds from sea water, and revealed a number of interesting features of the process. Most important, however, is the observation that while in specific instances oral (and even rectal) ingestion of dissolved nutrients may occur, uptake and utilization of these compounds is of primary importance only to the epidermis. Indeed, evidence has been accumulated which indicates that the epidermis is to a large extent functionally isolated from internal reservoirs of nutrients obtained through normal feeding activity, and must sustain itself primarily from the free organic compounds it can scavenge from the external media. Thus, epidermal uptake would complement feeding activity. It is a continuous process as opposed to the intermittent accumulation of foodstuffs in feeding, and is mainly significant to only a very small volume of cells with a very large exposed surface area. Such uptake however, may be essential to the survival of these cells.

The concept that marine animals can derive net benefit from dissolved organic compounds has not, however, achieved universal acceptance. Critics have noted the extremely low concentrations of dissolved amino acids found in "normal" sea waters (usually less than 1 micromolar total; *cf.*, Chau and Riley, 1966; Siegel and Degens, 1966; Webb and Wood, 1967), although reliable data is not available on the concentrations in the microenvironments of individual species. It has not been possible to determine how much energy the epidermal cells need expend to take up and retain nutrients from such dilute solutions. It is possible that the uptake mechanism could be coupled with other essential processes in the cells, metabolic alteration of the substrate, or physical adsorptive phenomena. For these reasons, the additional energy costs of transport to metabolism could be almost negligible. In any case, concern for energy budgets in the transport system does not at present appear to be a reasonable restriction to further inquiry.

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[1] Supported by NSF grant GB-14649 to Florida Presbyterian College and NSF Grant GB-5531 to the Friday Harbor Laboratories of the University of Washington.

The tracer techniques generally used to demonstrate uptake by a particular organism do not provide reliable information on the dissolved nutrients released back into the environment, and thus the net benefit from the uptake. This last point has been noted in a study by Johannes, Coward, and Webb (1969) on the commensal flatworm, *Bdelloura*. With the use of ion-exchange methods of analysis these workers observed that specimens of this species released into the media approximately 3 times as much amino acid as they took up. It should be apparent, however, that even if this observation were generalized to the great many species for which uptake of dissolved nutrients have been demonstrated, it would not lessen the importance of such uptake in the nutrition of specific tissues, such as the epidermis. Indeed, the nutrition of this tissue would be benefited if there were excretion of nutrients into its close proximity by other portions of the body! There is as yet no reason to believe that the observations of Johannes *et al.* (1969) should be broadly generalized.

Certainly many studies need to be completed on the uptake and release of free organic compounds by various species, and evaluation made of the net benefit derived from these nutrients. Such studies are only now becoming possible as new analytical techniques are developed. In the present investigation use has been made of gas-liquid chromatography to obtain initial information on the net uptake and release of amino acids by several species of starfishes.

The author is indebted to the Friday Harbor Laboratories of the University of Washington for providing facilities for this study.

MATERIALS AND METHODS

Ten species of starfishes from the Puget Sound area were used in the study. Represented were the following: *Pteraster tesselatus*, *Mediaster aequalis*, *Patiria miniata*, *Pisaster ochraceus*, *Dermasterias imbricata*, *Solaster stimpsoni*, *Stylasterias ferreri*, *Evasterias troschelii*, *Pycnopodia helianthoides*, and *Henricia leviuscula*. For the experiments, each specimen was placed in 750 ml of medium in a glass vessel partially submerged in fresh running sea water (11° C) on a sea table. The media consisted of freshly filtered (Millipore 0.45 micron) sea water to which known quantities of various pure amino acids were added. Twenty-five ml aliquots of the media were withdrawn at the beginning of the experiment and after 6 hours. These were placed in serum vials together with a measured quantity of β-alanine as an internal standard. The vials were frozen, lyophilized, and subsequently extracted with a 2 ml and a 0.5 ml portion of 90% ethanol containing 5% 1 N HCl. Solution of the amino acids was enhanced by placing the vials in an ultrasonic cleaning bath for several minutes. After centrifuging, the supernatant was slowly placed through a 1 × 14 cm column one-half full of regenerated Amberlite IR-120 H resin. The resin was then rinsed with 10 to 12 ml of double distilled water placed through the column in several aliquotes. The amino acids were eluted with 10 ml of repurified 7 N ammonium hydroxide. One ml of the eluate was placed in a small screwcap tube and dried under nitrogen in a sand bath at 100° C.

The amino acid samples were then quantitatively converted to N-triflouroacetyl n-butyl esters by the direct esterification method of Roach and Gehrke (1969). Separation was achieved by injecting approximately 7 microliters of the sample into a 4 foot glass column containing 0.65% ethylene glycol adipate on Chromosorb W in a Beckman GC-45 temperature programed gas chromatograph. This instrument was equipped with hydrogen flame ionization detectors and a disc integrator on the recorder. Instrument settings included 80° C initial temperature and a 32 minute 135° C rise after a 20% hold. Peaks were quantified by comparing their areas to that of the internal standard.

This method was sensitive to approximately 0.5 micromolar concentrations in the media (0.0125 micromoles per 25 ml sample) of the following amino acids: *l*-alanine, *l*-valine, glycine, *l*-isoleucine, *l*-leucine, *l*-proline, *l*-threonine, *l*-serine, *l*-cysteine, *l*-methionine, *l*-hydroxyproline, *l*-phenylalanine, *l*-aspartic acid, *l*-glutamic acid, *l*-tyrosine, *l*-lysine, and *l*-tryptophan. Sensitivity was primarily limited by the practical extent to which the various reagents used could be purified—especially the ion exchange resin.

TABLE I

Net change in amino acid content of media after 6 hours exposure to specimens (Initial concentration = 0.050 mM/l L-alanine)

Specimen	Wet wt. (g)	Volume medium (ml)	Initial *L*-alanine (μM)	Final *L*-alanine (μM)	Final total other a.a. (μM)	Principal a.a. released
Pteraster	178	750	37.5	0.5	0.5	GLY
Pteraster	249	750	37.5	tr.	0.0	
Pteraster	218	750	37.5	0.5	1.0	Gly, SER
Mediaster	49	750	37.5	20.0	0.5	GLY
Mediaster	19	750	37.5	29.5	2.0	GLY, SER, CYSH
Mediaster	73	750	37.5	15.5	1.0	SER
Patiria	184	750	37.5	7.0	0.0	
Patiria	146	750	37.5	15.5	1.0	GLY, GLU, TYR
Patiria	210	750	37.5	9.5	1.5	GLY, SER
Pisaster	361	750	37.5	4.0	1.5	GLY
Pisaster	381	750	37.5	3.0	0.5	GLY
Dermasterias	340	750	37.5	12.0	3.5	GLY, SER, ILEU, VAL
Solaster	272	750	37.5	0.5	5.5	MET, GLY
Stylasterias	300	750	37.5	tr.	2.0	GLY
Evasterias	810	1000	50.0	0.5	2.5	GLY
Pycnopodia	88	750	37.5	0.5	1.0	GLY, SER

RESULTS

Three main series of experiments were carried out. In the first of these an attempt was made to see if there is any net release of amino acids by starfishes into their media under relatively normal conditions. A low concentration (0.05 mM/l) of L-alanine was included in the media to verify that uptake mechanisms were functioning. The results are shown in Table I. In most cases only trace quantities (too low to be accurately measured) of glycine and occasionally other amino acids were detected in the media at the end of the 6-hour experimental period. The larger specimens took up most of the alanine present, and the smaller ones a large proportion of it.

In the second series, the starfishes were exposed to a mixture of amino acids, each initially present in a 0.05 mM/l concentration, to see whether the net effect

TABLE II

Net change in content of 12 amino acids in media after 6 hours exposure to specimens (Initial concentration = 0.05 mM/l; Volume, 750 ml)*

	Initial quantity	Quantity present after 6 hours					
Specimen wet. wt.		*Pteraster* 208 g	*Pteraster* 283 g	*Pteraster* 267 g	*Patiria* 191 g	*Patiria* 199 g	*Patiria* 174 g
Amino acid	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)
L-alanine	37.5	28.0	14.0	4.0	26.5	24.5	31.5
L-valine	37.5	28.5	18.0	5.0	26.0	26.0	35.5
glycine	37.5	35.5	40.5	48.5	26.5	23.0	27.0
L-*proline*	37.5	30.0	26.5	5.0	24.5	26.0	35.5
L-threonine	37.5	27.5	25.0	13.0	25.0	25.0	33.0
L-serine	37.5	22.5	21.5	8.5	23.0	22.0	27.0
L-methionine	37.5	19.5	10.5	4.0	19.0	20.0	26.5
L-phenylalanine	37.5	27.5	23.0	8.5	21.5	22.0	28.0
L-aspartic	37.5	19.0	22.5	15.5	18.0	17.5	14.5
L-glutamic	37.5	28.0	27.5	20.5	21.5	20.5	23.5
L-tyrosine	37.5	30.0	26.5	11.5	23.5	23.0	34.0
L-lysine	37.5	25.0	24.0	10.5	19.0	19.5	32.5

* No additional amino acids were detected in the media at the end of the period.

TABLE III

Net change in content of 12 amino acids in media after 6 hours exposure to specimens (Initial concentration = 0.05 mM/l; Volume, 750 ml)*

	Initial quantity	Quantity present after 6 hours					
Specimen wet. wt.		*Pisaster* 425 g	*Pisaster* 311 g	*Pisaster* 300 g	*Mediaster* 52 g	*Solaster* 138 g	*Pycnopodia* 94 g
Amino acid	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)
L-alanine	37.5	3.2	11.5	11.5	29.0	3.5	3.5
L-valine	37.5	1.0	7.5	9.5	28.0	34.5	2.5
glycine	0.0	6.5	9.5	16.0	1.5	1.0	18.0
L-proline	37.5	8.0	20.0	24.5	36.0	32.0	8.0
L-threonine	37.5	7.0	22.0	21.0	34.5	35.0	8.5
L-serine	37.5	3.5	16.0	17.5	37.5	15.5	5.5
L-methionine	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L-phenylalanine	37.5	1.5	11.5	13.0	35.5	29.0	25.0
L-aspartic	37.5	25.0	36.5	41.0	40.0	36.5	31.5
L-glutamic	37.5	29.0	40.0	37.0	42.0	34.5	35.5
L-tyrosine	37.5	4.5	18.5	19.5	39.5	33.0	5.0
L-lysine	37.5	4.5	17.0	15.5	38.5	39.0	6.0

* No additional amino acids were detected in the media at the end of the periods.

would be uptake or release to these compounds. In the first part of this series, 12 amino acids were included and the results are shown in Table II. While in every case there was a considerable net uptake of amino acids, in two of the experiments with *Pteraster* a net increase in glycine was detected in the media. In the second part of this series, glycine and L-methionine were omitted from the initial mixture (Table III). While *l*-methionine did not subsequently turn up in the media, significant quantities of glycine did. The overall effect again, however, was a very considerable net uptake of amino acids, even though in some instances the concentrations of a few slightly increased. It was further noted that in this series there was less uptake of L-alanine than when it was present alone in the first series. This result was expected on the basis of previously observed competitive inhibition phenomena in the amino acid transport system of starfish (Ferguson, 1968b).

Table IV

Net release of amino acids into media in the presence of 1.0 mM/l initial concentration of L-alanine (Volume = 750 ml; time = 6 hours)

Specimen	Wet. wt. (g)	Initial ALA (μM)	Final**										
			ALA (μM)	GLY (μM)	THR (μM)	SER (μM)	CYSH (μM)	MET (μM)	PHE (μM)	ASP (μM)	GLU (μM)	TYR (μM)	LYS (μM)
Pteraster	158	750	413	28.0	—	—	—	—	—	—	—	—	—
Pteraster	132	750	499	35.5	—	—	—	—	—	—	0.5	—	—
Pteraster	220	750	83	18.5	—	—	1.5	—	—	—	—	—	—
*Mediaster**	48	300	273	0.5	—	—	—	—	—	—	—	—	0.5
Patiria	175	750	624	4.5	—	—	—	—	—	—	—	—	—
Patiria	165	750	634	5.0	0.5	—	—	—	—	—	—	0.5	—
Pisaster	402	750	339	120.5	—	5.0	—	—	—	—	1.0	—	—
Pisaster	318	750	88	97.0	—	4.5	—	—	—	—	—	—	—
Pisaster	238	750	431	61.5	—	2.0	—	—	—	—	—	—	—
Solaster	130	750	491	13.0	0.5	—	—	0.5	—	—	—	—	—
Pycnopodia	180	750	167	132.0	0.5	3.0	0.5	—	0.5	0.5	0.5	—	—
*Henricia**	24	300	283	0.5	—	0.5	—	—	—	—	—	—	—

* Volume = 300 ml.

** Only amino acids observed in measurable concentrations are listed.

As the increased net release of glycine and the reduced L-alanine uptake observed in the second series of experiments was probably the result of competitive inhibition, a third series of experiments was carried out to see if net release could be enhanced utilizing this effect. In this series, a relatively high concentration (1.0 mM/l) of L-alanine was used to partially block reabsorption and thus more clearly reveal what amino acids (in the same transport series) are most involved in the release process. As may be seen in Table IV, glycine was by far the most significant amino acid released, although others also turned up. In most cases the net rate of uptake of L-alanine was at least an order of magnitude greater than the net total rate of release of the other amino acids.

While the same general results were obtained with all of the species used, some differences between them may be noticed in the data tables. Most of these differences are easily explained by the nature of the specimens themselves. Small types, such as *Mediaster* and *Henricia* took up considerably less of the dissolved amino acids than the larger species. Species with large surface areas, such as *Pycnopodia* and *Solaster*, were relatively efficient in their uptake in spite of the modest size of the specimens employed. *Pteraster* also took up amino acids very rapidly, but this species possess a unique aboral chamber which it actively ventilates.

DISCUSSION

In all the cases examined in this study, the net flux of amino acids was overwhelmingly inward, as one would expect it to be if these animals derive net benefit from dissolved environmental nutrients. The quantity taken up was considerably larger when the starfishes were exposed to greater concentrations, but a higher proportion was taken up when lower concentrations were employed. This result is compatible with those of previous studies of starfish transport mechanisms (Ferguson, 1964, 1968b).

It must be noted, however, that the least concentration used, 0.05 mM/l L-alanine, while very low in absolute terms, was still quite a bit higher than that found in "natural" sea water. This condition is evident in the fact that clean natural sea water was used in the formulation of the media used, and it did not contain sufficient concentrations of amino acids to be significantly registered by the analytical techniques employed. It would seem, though, that concentrations such as those used could easily occur in the microenvironments of the animals, particularly during feeding activities—but experimental verification of this is as yet lacking.

In spite of these practical difficulties, the experimental situation employed does provide considerable insight into the nutritional processes. The net inward flux is so great even at the lowest concentrations used, that this effect could certainly be extrapolated to sea water much less rich in nutrients. Furthermore, probably as a result of the relief of the competitive inhibition of the transport system, net release of amino acids appears to become more limited as there is a decrease in the concentration in the media of amino acids of the same transport group. Thus, the present results definitely support the concept that starfish can receive net benefit from the dissolved nutrients that may be found in their environment.

There is no question, however, that these animals are somewhat "leaky" systems. When the transport mechanism is inhibited they lose the ability to retain their natural pools. It would appear, then, that the transport system has as equal importance in retaining endogenous amino acids as in taking up exogenous ones. Any factor influencing the transport system, such as reduced salinity, temperature, inhibiting compounds in the sea water, *etc.*, would be expected to influence the composition of the internal amino acid pools, and thus profoundly effect the physiological state of the animals. Previously, this consequence has not always been fully appreciated.

The specific amino acids released, the primary one being glycine, seem to be those that predominate in the tissue pools. Preliminary analyses of the free amino acids found in the body walls of these species indicate that glycine is often present at over 100 times the concentrations of the other major amino acid components.

This high glycine concentration has also been noted in the few published accounts of the free amino acid content of other starfishes (*e.g.*, Giordano, Harper and Filice 1950; Jeuniaux, Bricteux-Grégoire and Florkin, 1962). The significant amounts of *l*-serine and other amino acids that turned up in the media of the third series of experiments also appear to correlate with the presence of these compounds in the tissue pools of the various species, but a more comprehensive study of the free amino acids of these species has not yet been completed.

It is becoming increasingly evident, from the results of this study and others in the literature, that the free amino acid pools maintained in the various tissues of these animals are in a delicate balance with chemical and physical factors of the external environment and, doubtless, the internal physiological state. Practically nothing is yet known about the interaction of these parameters in the microenvironments of the various species throughout the seasons of the year or the life history of the animals. Obviously this whole area must be explored in future research if a true picture of the biology of these animals is to be developed.

SUMMARY

1. Net uptake and release of dissolved free amino acids was measured in experiments with 10 species of Puget Sound starfishes, utilizing gas liquid chromatography.

2. When specimens were placed in filtered sea water with 0.5 mM/l L-alanine, most of the amino acid was removed within a 6 hour period, and no more than trace quantities of other amino acids appeared in the media.

3. When specimens were placed in filtered sea water with 0.5 mM/l concentrations of 12 amino acids, there was considerable net uptake of all the amino acids except glycine, which in two cases with *Pteraster* was further released into the media.

4. In a similar experiment in which glycine and L-methionine were omitted from the mixture, there was significant net release of glycine but not *l*-methionine, simultaneously with considerable uptake of the majority of the other 10 amino acids in most cases.

5. When the uptake mechanism for neutral amino acids was partially inhibited by including 1.0 mM/l L-alanine in the media, a fairly large efflux of glycine and occasionally a much lesser amount of *l*-serine and other amino acids was detected. The amino acids released by the various species appeared to correlate with those maintained free in their tissues.

6. The results of the study support the concept that starfish can receive net benefit from dissolved nutrients in their environments. They further indicate that the transport system for taking up amino acids is also significant in the retention of those amino acids already in the metabolic pools.

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ON THE HEART OF THE ORANGE TUNICATE, *ECTEINASCIDIA TURBINATA* HERDMAN

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Members of the genus *Ecteinascidia* belong to the Subphylum Tunicata. This subphylum is also referred to as the Urochordata (Urochorda), and the animals are commonly known as ascidians or sea-squirts. As a group, they are highly successful, diverse and specialized (not degenerate) protochordate members of the Phylum Chordata (Barrington, 1965). *E. turbinata* Herdman grows at various depths on exposed surfaces in dense, almost spherical, usually pinkish orange to reddish orange clusters of various diameters (colonies measure from about one to 7 inches), made of many sessile individuals. Many of the individuals in a colony are connected by short, delicate, branched stolons. *Ecteinascidia* belongs to the largest of the 3 classes of the Tunicata: to the Ascidiacea (Berrill, 1932; Van Name, 1945).

A very large literature is available on the hearts of many species of tunicates and most of the pertinent information on the anatomy and physiology of this organ has been well-reviewed by Skramlik (1929, 1938), Millar (1953), and Krijgsman (1956). The primary and perennial interest in the hearts of tunicates is the periodic reversal in the direction of the heart beat in all species of these quite diverse animals. The physiological usefulness (if any) of the periodically reversing circulation still remains unknown. Further, none of the various explanations of periodic reversal is entirely satisfactory (Mislin, 1969). The two major theories of causes of heart beat reversal in tunicates are, first, that back pressure gradually builds up within the vascular system to inhibit the active pacemaker and, second, that the active pacemaker "fatigues." Krijgsman (1956) concluded that regular reversal of pulsation could not be accounted for by the back pressure theory. He proposed that periodic fatigue and subsequent restoration of heart pacemakers offered a better explanation. Nevertheless, the fatigue theory is ill-defined and poses more questions than it solves. The phenomenon of heart reversal is not unique to tunicates since it is known to occur in at least 7 orders of insects (Jones, 1964) and has been observed in blood vessels of both vertebrates and invertebrates (Azariah, 1965; Mislin, 1969). Beklemishev (1969) refers to heart reversals in Nemertinea and in *Amphioxus*.

Although the hearts of many species of tunicates have been studied in varying detail, that of *Ciona intestinalis* has been studied more extensively and in far more detail than that of any other single species. Consequently, information on *Ciona* tends to dominate and outweigh that on other species. The present paper deals with observations made each summer over a five year period on the general anatomy and physiology of the heart of *Ecteinascidia turbinata* Herdman, 1880.

MATERIALS AND METHODS

Most specimens were collected from the pilings off Longbird bridge, just past the entrance to Castle Harbour in Bermuda. Usually within an hour of collection the colonies were secured at the bottoms of tanks of flowing sea water, some distance away from the slow steady inflow. Colonies also did moderately well when kept in large beakers of sea water which was changed each day. The water temperature varied from 25 to 28° C. Unless stated differently, healthy-looking tunicates were carefully torn from a colony with fine forceps and placed with their right sides uppermost in 10 ml of fresh sea water in small glass dishes. Normally, the animals live with their siphons uppermost, their posterior stolons attached to the substrate. When removed from the colony, the animals can move about considerably by currents set up by their siphons. Animals which were badly silted or in obviously poor condition were automatically discarded.

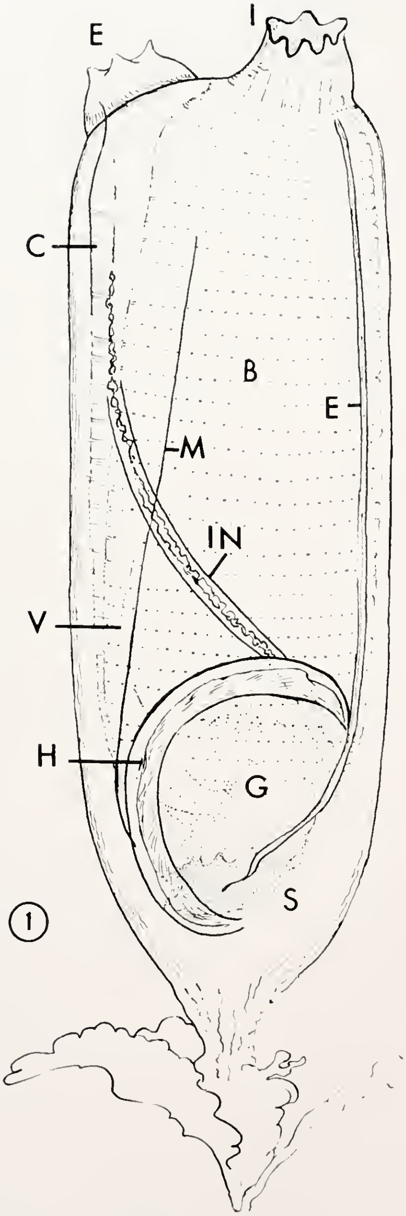
As in other tunicates, a consecutive set of anterior to posterior contractions (those beginning at the anterior or hypobranchial or branchial pole) is termed *advisceral beating* or an advisceral cycle, and the set of posterior to anterior pulsations (those beginning at the posterior or visceral pole) is called *abvisceral beating.* The encasing pericardium itself was never observed to contract in any of the tunicates examined in this study.

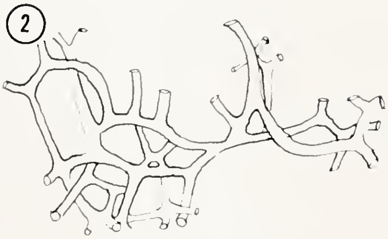
In general, only complete advisceral and abvisceral cycles were recorded. That is, counts of heart beats were begun only after observing a reversal and then all the beats in a given direction (pulsation series or cycle) were counted. Whenever possible (about 95% of the cases), a minimum of 3 complete consecutive pulsation cycles in both directions were recorded for each animal used. Where the size of the tunicates is not given, the animals measured at least 25 mm in body length (from the top of the siphons at the anterior end to the approximate beginning of the stolon). In most surgical experiments, the animals were not observed for more than 2 hours. Semi-isolated and isolated hearts were sometimes observed for 1 to 2 hours only, but often for less than 1 hour.

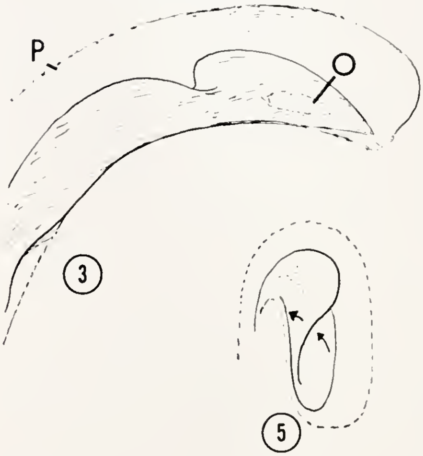
Results

1. *The anatomy of the heart*

The heart is a long, relatively large, slightly twisted, dorso-ventrally oriented, unchambered, C-shaped, tubular vessel on the lower right side of the tunicate (Fig. 1, H). The heart opens into the hemocoele at each end (Berrill, 1961). The hemocoele is a large space of cavities and discrete vessels. The vessels were never observed to contract. Although difficult to see in both living and fixed material, single slit obovate ostia (Fig. 3, O) occur at each pole of the heart. The heart lies underneath the transparent tunic and is external to the large branchial basket (Fig. 1, B). The heart is tapered abruptly at both ends. Near the center of the heart is a short permanent constriction. When seen ventrofacially from the endostyle, the anterior apex is obovate and ventrally directed. The anterior pole terminates near the posterior end of the pale yellowish endostyle (Fig. 1, E). The posterior end of the heart is located internally, more deeply within the body than the anterior end, and is attached some distance to the right of the centrally-located, dark yellowish-brown stomach (Figs. 1 and 4, S). The stomach opens into the intestine on the ventral side. The intestine proceeds ventrad and then spirals around to the dorsal side, outside of the branchial basket (Fig. 1, IN). The tip of the posterior pole of the heart and its attachments was never seen as clearly as those of the anterior pole.







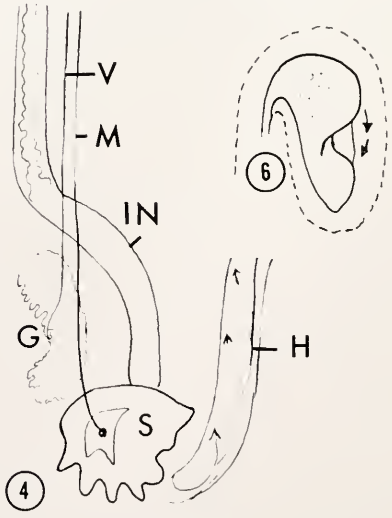


FIGURE 1. Semi-schematic view of adult *Ecteinascidia turbinata* as seen from the right side and showing the antero-dorsally located excurrent siphon (E), the antero-ventrally located incurrent siphon (I), circular smooth muscle bands of the mantle (C), the large branchial (or pharyngeal) basket (B), the long, slender, ventral endostyle (E), the mucus column (M), the intestine (IN), the vas deferens (V), the heart (H), the gonads (G), and the stomach (S).

FIGURE 2. Non-contractile vessels within the tunic (semi-schematic).

The heart is completely encased in a large clear pericardium (Fig. 3, P). The pericardial cavity is probably the only remaining vestige of a coelom in tunicates (Barrington, 1965). While a definitive pericardial body was never observed, a large clump of hemocytes was sometimes seen within this cavity. During the formation of the heart, the pericardium folds inward and the site of the infolding subsequently serves as the sutural attachment along nearly the whole length of the heart (on its internal or left side, along the posterior margin). This long suture is the raphe (Fig. 7, R). *Ecteinascidia* totally lacks an epicardium, according to Lefevre (1897).

A large variable space exists between the anterior face of the heart and the anterior surface of the pericardium (Fig. 3). The heart is made up of a complexly wrapped single layer of long, thin, cross-striated muscle fibers (Fig. 7, H). The latter have a distinct spiral orientation. Complex folds are seen at the anterior end of the heart. When the isolated heart is examined in a fresh sea water whole mount with phase contrast optics, clear isotropic and grey anisotropic bands in the different fibers are seen to be aligned and are approximately equal in size. Sharp Z bands are present. A single cardiac muscle strand is made up of many nucleated cells connected to each other by several delicate, longitudinally orientated and banded rami. The muscle nuclei are large and ovoid, and each has a single conspicuous nucleolus. In all of the material examined, there was no indication that cardiac muscles degenerate or are cast off into either the heart lumen or the pericardial cavity [as in *Ciona* (Millar, 1953)].

In freshly dissected whole mounts seen with phase microscopy, long, fine (about 0.5 μ in diameter in a large tunicate), phase-dark nerve fibers were observed in delicate weft-like nets in the wall of the pericardium and around the heart (Fig. 8, N). These fibers lacked nuclei and had many small bead-like swellings or tiny nodes. Some of the many delicate arborizing branches were found within the myocardium itself. The nerve network was not localized or concentrated at any particular region of the heart. Some branches of the network could be traced to the ganglion located between the siphons at the anterior end of the animal (="brain" ganglion). The nerve network did not stain when either intact tunicates or fresh whole mounts of the heart were kept in various concentrations of methylene blue in sea water for long periods.

2. *General behavior of the intact heart*

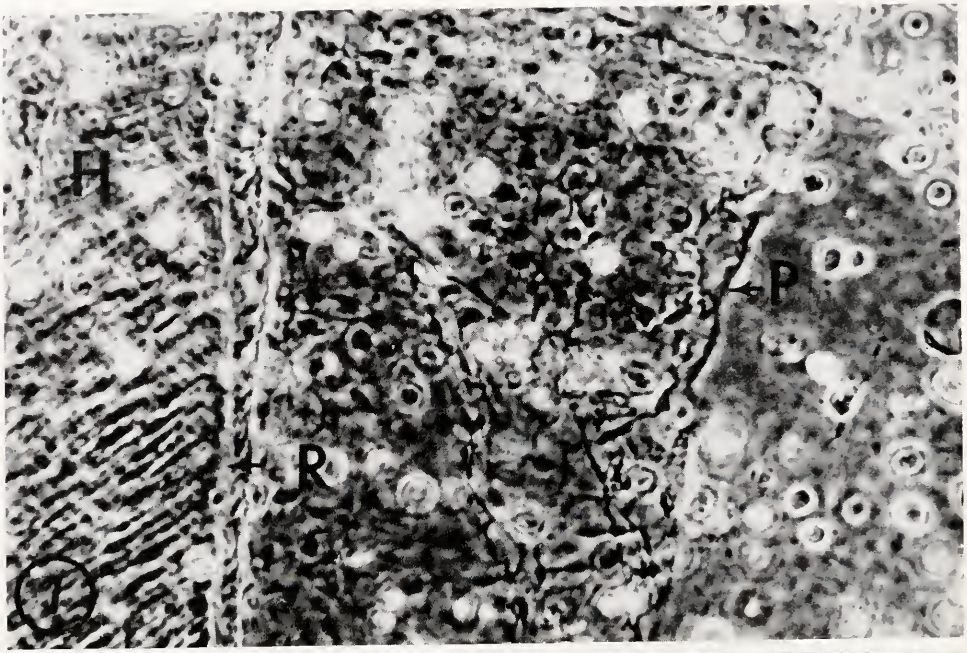
The heart of intact, highly responsive and healthy-looking tunicates exhibited a wide range of behavior. The most striking phenomenon was the condition of

FIGURE 3. The anterior end of the heart showing the large non-contractile pericardium (P), and the slit-like ostium (O). The ostium is shown more clearly than it is ever visible in fresh material (semi-schematic).

FIGURE 4. Dorsal schematic view of a portion of *Ecteinascidia* showing the vas deferens (V), mucus column (M), intestine (IN), gonads (G), stomach (S) and the heart (H). Note that the posterior pole of the heart is located alongside the stomach. Arrows indicate the pathway of peristaltic contractions during abvisceral beating.

FIGURE 5. Antero-ventral view of the anterior end of the heart during advisceral beating. Note that the heart folds dextrad (arrows, semi-schematic).

FIGURE 6. Antero-ventral view of the anterior end of the heart during abvisceral beating, just as the contraction wave is reaching the anterior pole. Note that the heart is again folding dextrad (arrows).



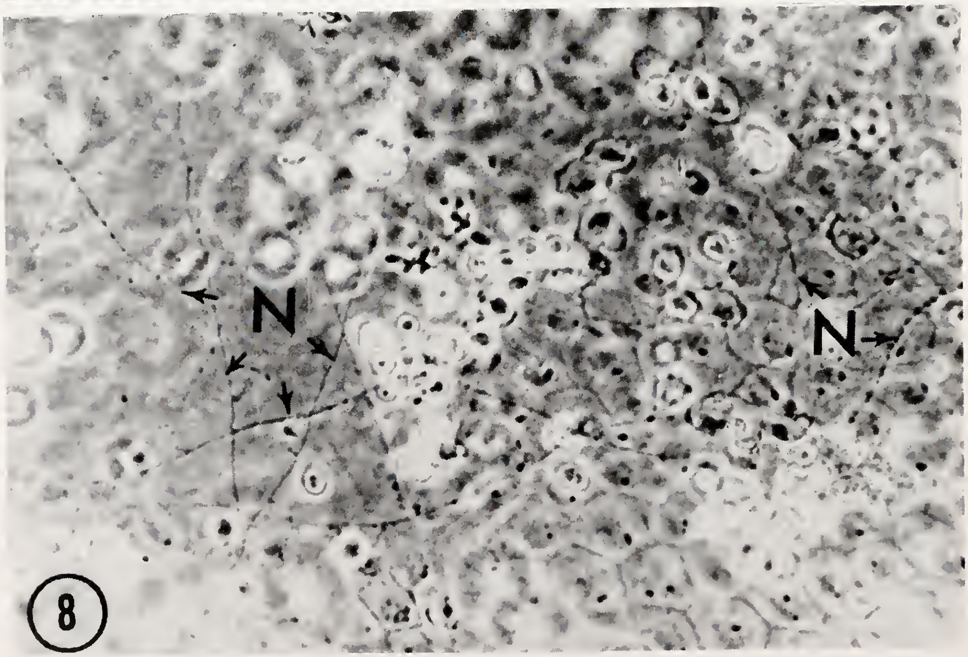


FIGURE 7. Unstained, phase-contrast appearance of a portion of the heart (H) of *Ecteinascidia turbinata* showing the raphe (R), and the pericardium (P). The numerous scattered globular structures are hemocytes.

FIGURE 8. Unstained phase contrast view of nerve network (N) within the vicinity of the heart; note the fine beaded appearance of the nerves.

prolonged cardiac arrest in diastole. While this condition was commonly found in tunicates that had just been isolated from a colony, some hearts in diastolic arrest were observed continuously for as long as 2 hours without the occurrence of any pulsations. Arrested hearts were seen in many fully relaxed, normal-looking tunicates with active siphons. A few of these individuals were examined once a day over a period of several days so that in some cases, at least, the condition of cardiac arrest may be very prolonged in some otherwise healthy-looking and highly responsive animals (their siphons giving quick responses to touch). In most cases, if the heart was not beating within the first hour, it was not re-examined.

In some tunicates, the condition of diastolic arrest was followed by a state of prolonged erratic beating of the heart. Erratic beating was particularly noticed in animals which had just been isolated for study. In the commonest type of erratic heart activity, the heart would pulse one or two times in one direction or the other and then stop and start again at very irregular intervals. Prolonged erratic heart beats were observed in numerous contracted and relaxed tunicates. Erratic activity also included those cases in which one or both poles of the heart would contract locally: that is, heart contractions were not conducted over the full length of the cardiac tube. Local polar contractions were often rhythmic and sometimes alternated between poles, but generally these localized polar contractions were highly arrhythmic and not specifically alternative in character Erratic cardiac activity also includes those cases where contractions would begin at both poles simultaneously or nearly so and be conducted towards the center of the tubular heart. Such bipolar beating was not a commonly observed phenomenon. More rarely, a third condition was observed in intact tunicates; a single contraction would begin in or near the constricted center of the heart and sweep outwards towards the poles.

Over a 5 year period, the condition of prolonged cardiac arrest was found in about 5% of the healthy-looking individuals examined. The condition of prolonged erratic beating was seen in 2% to 39% (average of 18.4%) of the individuals. The majority of the tunicates had hearts which exhibited prolonged rhythmical beating. Rhythmical beating sometimes followed a period of prolonged erratic cardiac activity. Rhythmical beating of the heart in intact *Ecteinascidia* proved to be highly variable. No differences could be seen between the degree of distension of the heart within the pericardial cavity and any specific cardiac activity in intact animals.

3. *Heart contractions, reversals, and circulation of the hemolymph*

The heart's contractions and circulation of the hemocytes within the clear hemolymph can be seen in its finest details and with brilliant clarity in most intact *Ecteinascidia*. When the heart beats, a peristaltic wave normally begins only at either the anterior or posterior pole. Normally, the contraction waves appear as large, full, ballooning billows that sweep over the tube. On one occasion a tightly constricted heart tube was seen in an intact healthy-looking individual; this animal's heart beat and reversed very rapidly. Later, this animal's heart expanded fully, and did not then reverse as frequently.

Heart contractions are conducted in definite twisting spiralling patterns over the length of the heart (see, for example, arrows in Fig. 4). During contractions, the heart folds dextrad (see Figs. 5 and 6). Only a relatively small portion of the heart tube (about one-twelfth of the tube length) is partially (but never completely) occluded during the passage of a constriction wave.

As in all other tunicates thus far studied (see Krijgsman, 1956), after a variable number of beats the direction of the contractions changes from one pole to the other. In *E. turbinata* these reversals begin in the late embryo (they are not synchronized with those of the mother) and continue throughout the life of the individual. Although repeated specific synchronized reversals of the heart in two connected individuals have been seen among connected samples of *Ecteinascidia conklinii typica*, synchronized reversals have never been observed between connected individuals of *E. turbinata*.

Reversal in direction of peristaltic waves occurred with and without pauses of generally short but highly variable duration between sets of beats. During a reversal pause, the heart tube may passively swell and subside slightly, and very fine ripples may pass over the non-contracting vessel. Pauses between consecutive beats in both advisceral and abvisceral directions are quite common before the heart beat reverses. As in *Ciona*, the heart rate is usually faster shortly after heart reversal than near the end of a pulsation series.

Towards the end of a given cycle, the contractions tend to become slower. One or more final beats may still occur before the heart stops and reverses. Many times the heart stops very suddenly without slowing down, and reversal in direction occurs after a very brief pause. Pauses before reversals varied enormously (from about 0.5 seconds to far more than 15 seconds); no definite relationships between these pauses and the size of the animals were noted. In a few individuals, at the very end of a cycle, there may be an obvious sudden brief backflow of hemolymph at the excurrent ostium. The actual backflows seen at the anterior end of the intact heart never exceeded one-seventh the length of the heart tube.

When heart pulsations cease, the entire circulation in the whole animal also instantly ceases. In intact animals during rhythmical beating, hemocytes can be easily seen flowing in dense spiralling streams into and out of the heart through the slit ostia at each pole. The heart never ejects all of its hemolymph into the extracardiac system *in vivo* at any one contraction. When the heart and pericardial sac are removed from the animal without any mechanical injury to these structures, they both always collapsed and were largely empty of hemolymph. The heart is filled from ostia at the poles; how the pericardium is filled is completely unknown.

During advisceral beating, the hemolymph moves away from the animal's incurrent (ventral) siphon and passes posteriorwards in two major currents down the sides of the endostyle. The hemolymph in the many small branchial vessels circles around the body and flows into the endostylar currents. Numerous close observations of the vessels in the test and branchial basket never showed them to contract in any way. Hemolymph flows from the posterior end of the heart and is directed into at least four channels. One of these streams goes around the non-contractile stomach towards the gonads on the left side of the animal. Another stream passes upwards towards the excurrent (dorsal) siphon and forms distinct rivulets on either side of the non-contractile intestine. Still another stream moves into channels along the esophagus and mucus column and proceeds towards the siphons and branchial vessels.

During abvisceral beating, one large current of hemolymph flows away from the excurrent siphon posteriorwards and passes over the stomach in a direction opposite to that during advisceral beating. Hemolymph streams pass posteriorwards over the intestine. Hemolymph spurts out of the anterior end of the heart and anteriorly-directed currents flow up to the incurrent siphon. At the same time, one large posteriorly-directed current of hemolymph flows on either side of the endostyle.

4. *Variability of the heart rate*

By far the most striking feature of the contractions of the heart in intact *E. turbinata* was the extreme variability in the number and duration of both advisceral and abvisceral beating. The amount of hemolymph circulated during a particular pulsation series would therefore vary enormously even in the same individual. A long series of studies were made to determine some of the possible sources for the great variability of the heart rate in normal animals.

A few records were obtained from 16 newly emerged tadpoles by placing them between a slide and coverslip in a drop of fresh sea water. No heart beats could be detected over a 5 minute interval in three of the individuals. The hearts stopped beating in 4 other individuals which were being examined. The heart rates of the tadpoles examined were highly variable. The number of beats occurring in both advisceral and abvisceral cycles were obtained along with the duration of each. The number of beats in advisceral cycles varied from 1 to 56 and averaged 14.3. The duration of this cycle varied from 5 to 67 seconds, with a mean of 24.6 seconds. The number of beats in the abvisceral cycles varied from 2 to 87, with an average of 21.0 beats. The duration varied from 4 to 222 seconds, with a mean of 42.5.

A single large (25 mm) healthy-looking tunicate was selected from a colony on the day of its capture and was arranged in a vertical position with its siphons uppermost at the bottom in a 100 ml graduate and was examined continuously over a period of 7 hours under varying amounts of fresh sea water. No marked differences in the number of heart beats and their duration were apparent in this individual in differing volumes of sea water. Similar results were obtained in contrasting heart rates of another individual kept first in 250 ml then in 50 ml of fresh sea water over a 3 hour period. The next day this same tunicate was gently removed from the 100 ml graduate and placed on its right side in a small dish containing 10 ml of fresh sea water. After this change, the tunicate was examined: its heart had stopped beating. The heart began to beat erratically and did so for at least 10 minutes. After this, the heart began to beat rhythmically again at a rate not strikingly different from that in the vertical position in 90 ml sea water.

Complete heart rates were taken from 194 individuals that ranged in size from about 1.5 to 40 mm in length. No clear-cut relationships or trend between the size of the tunicates and heart beat and its variability could be seen.

Although the heart rate of *Ecteinascidia* is highly variable between individuals, the heart in 5 groups of 261 animals averaged 60 to 77 beats in either ad- or abvisceral direction (overall mean 69.6 ± 1.6 beats) each summer over a 5 year period. The average duration of beating in either ad- or abvisceral direction ranged from 87 to 114 seconds (overall mean 99.5 ± 2.6 seconds.) The overall advisceral rate for each of 5 summers was 71.4 ± 2.7 beats in 102.5 ± 4.4 seconds and the overall abvisceral rate was 67.9 ± 2.5 beats in 96.4 ± 4.1 seconds. Thus, there is no significant difference (95% confidence limits) in rates from either direction. In different words, neither the anterior (=hypobranchial) nor the posterior (=visceral) center is dominant in normal intact healthy *Ecteinascidia.*

Daily mean heart rates from 70 relatively large tunicates (20 to 30 mm length) from a single colony were analyzed in relation to days (1–12) they were kept in the sea table. The mean advisceral rate was 66.6 ± 5.0 beats in 93.1 ± 5.0 seconds, and the mean abvisceral rate was 62.3 ± 4.4 beats in 87.5 ± 5.0 seconds. No obvious trends in the heart rates were apparent relative to time after captivity.

Three tunicates were placed in small dishes containing 10 ml of sea water and three complete advisceral and three complete abvisceral rates were recorded consecutively every hour on the hour for a 24 hour period. The sea water was renewed at aperiodic intervals. The first readings were compared with those in subsequent hours. The heart rates were more variable during the first hour than at any time thereafter among all three individuals. After the first hour, the heart beats and their duration tended to be remarkably uniform within a single individual (most rates were within 10 beats of each other per individual). Great differences were still apparent, however, between heart rates among the three individuals. Nevertheless, in small amounts of unchanged sea water, the hearts maintained a strikingly uniform number of beats for many hours. In all three tunicates the heart rate began to increase around the tenth or eleventh hour: that is, the number of beats and their duration in both advisceral and abvisceral cycles definitely increased with time, and this increase was well-established around the twelfth hour. In unchanged sea water the heart contracted more slowly and beat longer in each direction during the last 12 hours than during the first 12 hours.

Three fresh tunicates were then placed in small dishes containing 10 ml of sea water which was constantly changing at a rate of 2.5 to 17 ml per minute, and heart rates were taken hourly over a 24 hour period. The number of heart beats in each tunicate decreased after the first hour. The number of beats was strikingly uniform within and between all three of these individuals for about 20 out of the 24 hours. While the number of beats was not highly variable, the duration of both cycles was extremely variable. During the last 4 hours, the duration of both cycles definitely increased. In marked contrast to the tunicates in unchanged sea water, the number of heart beats did not significantly increase at any time during the 24 hour period when the sea water was constantly flowing. Although the duration of both cycles increased in changing sea water, this increase was much less and occurred considerably later than in tunicates kept in unchanged sea water.

In summary, the variability of the heart rate of *E. turbinata* is very great during the first hour of observation. This variability is not related to orientation of the tunicate in the observation chamber, to the volume of sea water in the observation chamber, to the body length, or to the time held in the sea table, or to the use of animals in obviously poor physical condition.

6. *Studies on heart reversals in intact animals*

In manipulating tunicates for heart rate studies, it had been qualitatively observed that handling frequently was associated with erratic beating and with greatly increased reversal rates. Therefore, two experiments were made to test the effects of external pressure on the heart rates of intact tunicates. In the first experiment 5 tunicates were used to obtain pre-test heart rates in fresh sea water and then a uniform weight was positioned on the body to exert a strong continuous pressure on the upper halves of the animals, considerably anterior to and outside of the heart region. This pressure caused violent contraction of the tunicates and tight closure of both siphons, resulting in an increased pressure in the hemocoele. A series of heart rates were taken during the period of continuous pressure and then the weight was suddenly lifted with a consequent sudden marked decrease in pressure in the hemocoele, and another series of heart rates were taken as soon as possible after the pressure was released. As summarized in Table I, strong pressure on the anterior ends of intact tunicates increased the already great inherent variability of cardiac data.

TABLE I

Mean heart rates with standard errors from Ecteinascidia turbinata *before, during, and immediately after strong external pressure on the anterior ends of five animals*

Treatment	Advisceral rates		Abvisceral rates	
	Beats	Seconds	Beats	Seconds
No pressure	93.1 ± 7.9	115.1 ± 18.5	84.9 ± 11.7	112.2 ± 12.4
Strong pressure	68.5 ± 16.3	85.2 ± 20.1	59.2 ± 10.8	88.5 ± 15.2
After pressure	96.1 ± 15.6	100.7 ± 23.7	73.7 ± 17.3	94.8 ± 22.3

Five tunicates were used to study the effect of localized pressure on the heart in intact animals. A blunt probe was firmly and strongly pressed over the anterior end and/or over the center of the heart immediately after the first 5 beats of either the advisceral or abvisceral cycle. The probe could not be placed or maintained firmly or specifically directly over the abvisceral end because it is located much more deeply within the body of the animal. The data in Table II show that external pressure over the heart tube greatly increases the rate of reversals in intact animals. After external pressure, the hearts averaged 18.5 beats in 32.4 seconds before reversing in direction. It was observed that even during strong external pressure over the anterior center, advisceral beats would still arise therefrom.

The siphons, along with the "brain" ganglion (which is located anteriorly between the siphons), were removed with single scissor cuts from 5 tunicates shortly after taking a series of pre-test heart rates, and heart rates were recorded immediately afterwards and one hour and 3 hours afterwards. The heart rates were highly variable within and between individuals before (means of 66.5 ± 9.5 beats in 93.4 ± 13.7 seconds) and after siphonal removal (means of 75.5 ± 3.3 beats in 107.8 ± 5.8 seconds). The hearts continued to beat and reverse for at

TABLE II

Effects of external pressure after the first 5 beats in a given direction on reversals of the intact heart of Ecteinascidia turbinata

Animal No.	Trial No.	Heart rates before reversal after pressure on					
		Anterior center at beginning of advisceral cycle		Center of heart at beginning of advisceral cycle		Anterior center at beginning of abvisceral cycle	
		Advisceral		Advisceral		Abvisceral	
		Beats	Seconds	Beats	Seconds	Beats	Seconds
1	1	22	33.8	21	31.2	10	14.2
	2	28	42.0	—	—	19	25.0
2	1	22	42.4	30	53.6	31	58.2
	2	25	44.2	—	—	32	60.0
3	1	11	23.2	18	35.0	19	35.0
	2	16	32.2	—	—	16	30.6
4	1	9	17.0	19	30.0	18	33.0
	2	8	13.8	—	—	17	28.4
5	1	14	25.6	14	25.2	19	33.0
	2	8	14.8	—	—	17	29.6

least 24 hours after siphonal (and brain) removal from a series of representative tunicates.

Ten tunicates were used to study the effects of cutting through the approximate center of the intact heart. After a series of pretest rates were taken, each heart was cut completely through with a single small scissor cut. The incision always led to sudden brief loss of a variable (usually large) amount of hemolymph which clotted almost instantly. The wound was sealed with great rapidity and then blackened. This rapid clotting would seriously interfere with accurate blood volume estimations based on attempted exsanguination. The hearts always stopped in diastole the instant they were cut with the scissor blades. Unlike *Ciona,* the cut hearts did not swell. Although small and large amounts of hemolymph could be removed from the heart *in situ,* no marked differences in the diameter of the intact heart could thus be consistently produced in *Ecteinascidia* (unlike *Ciona*). The heart was never observed to distend to such an extent as to fill the large pericardial cavity in any of numerous surgical experiments. After cutting the center of the heart, the tunicates were observed for periods ranging from 25 minutes to 20 hours; the average time was 105 minutes. The hearts remained in diastolic arrest for 1 minute to more than 2 hours, for an average of about 20 minutes. In 80% of the tunicates the posterior half began to beat before the anterior half, and, in some cases, was the only half to beat at all. When the anterior half did beat, the contractions were usually less vigorous than those in the posterior half. In general, the contractions were only towards the cut center. In a few cases, beats were initiated from the site of the incision. Reversals only rarely occurred in centrally-cut hearts. When contractions occurred in both anterior and posterior halves in the same tunicate, they were very rarely synchronized. Centrally-severed hearts were never observed to resume truly normal rates with periodic reversals like those of untreated controls.

When the heart was removed along with the pericardium in a series of tunicates, it collapsed. While a few pulsations sometimes persisted from one or both poles of the isolated heart, the contractions were highly abnormal in character. Usually individual muscle fibers would contract either in unison and/or asynchronously with other heart muscle fibers. Most attempts at filling the excised heart and/or the pericardial sac with sea water were not successful. Where the excised heart and/or pericardial sac could be inflated to various degrees, however, either the heart never beat at all or it pulsed very erratically and abnormally. That is, the contractions were not smooth, large, full, billowy waves, but peculiar vibratory contractions of small amplitude. Periodic mechanical stretching and releasing of isolated and semi-isolated (exposed) heart preparations did not induce beating in already arrested hearts and generally appeared to stop erratically beating hearts. Gentle probing or touching of quiescent isolated or semi-isolated (exposed) hearts generally did not elicit rhythmic contractions, although one or many individual cardiac strands would contract.

Discussion

The present studies on *Ecteinascidia* are interpreted to mean that under optimum conditions, the heart of a given individual will beat and reverse at a uniform, if highly idiosyncratic rate, whether the animal is an embryo, a tadpole, an immature zooid or a mature adult. If the animal is mildly disturbed by mechanical stimuli, then its heart rate often rapidy becomes highly variable with a tendency to be accelerated for about one hour. If the disturbance is stronger, then the heart beats erratically for a variable but usually long period, and if the disturbance is still greater, then the heart stops beating completely for a variable but generally long time. What could be the advantage of no circulation during stress? Although one advantage might be that it would reduce hemolymph loss, the hemolymph rapidly clots *in vitro* and quickly seals even large wounds. It would be useful to know if cessation of circulation would aid wound healing.

Ecteinascidia may look perfectly normal, have its siphons open and be highly sensitive to touch, and live for a long time without a pulsating heart (*i.e.,* with no circulation of the hemolymph). That a relatively large number of such individuals may have a very erratic circulation of the hemolymph indicates that a heart and efficient rhythmical circulation of the blood are not essential. This interesting condition is like that described for certain insects (Jones, 1964).

Since the fine network of nerves within the pericardium and myocardium of *Ecteinascidia* is so diffuse, the nerves cannot be acting directly and specifically on the poles from which all of the beats normally arise. Thus, it is believed that the nerve network cannot be associated with the initiation of heart beats. The *Ecteinascidia* heart definitely lacks nerve cells ("cardiac ganglia"). Almost all workers agree that the tunicate heart possesses essentially myogenic pacemakers. Although arthropods are very often said to have "neurogenic" hearts, this is certainly not true for the insects, which represent the largest class of these animals (see, for examples, Jones 1954; McCann, 1961; and Miller, 1969). Most insects are believed to have innervated myogenic hearts (Jones, 1964). Thus, the *Ecteinascidia* heart has more in common with the typical insect heart than with the typical neurogenic hearts of *Limulus* (Carlson, 1905) and many Crustacea (Maynard, 1960).

It is assumed that the extensive nerve network in and around the heart has a marked influence on the heart beat and reversals in *Ecteinascidia*. Perhaps it is the source of the extreme sensitivity of the heart: the heart can very quickly accelerate, decelerate, and stop and start again. Under optimum conditions, it is probably exquisitely well-regulated, although this system is very easily disrupted. Since the *Ecteinascidia* heart continues to beat and reverse normally when the "brain" ganglion has been removed, the network cannot be controlled or regulated by this ganglion.

Specific findings on *Ecteinascidia* can be compared best to those available for *Ciona*. While the *Ecteinascidia* heart is well-innervated, there are conflicting reports on the question of cardiac innervation in *Ciona*. Thus, Krijgsman (1956) did not believe the *Ciona* heart was innervated. Millar (1953), Ichikawa (1966) and Anderson (1968) did not find cardiac nerves in their studies on *Ciona*. On the other hand, Alexandrowicz (1913, page 373) observed many fine cardiac nerves in the myocardium of *Ciona*. Although Florey (1951) specifically cites the work of Fedele (1923a; 1923b; 1927) in connection with his proposal that cardiac nerves in *Ciona* were cardio-inhibitory, I can find no statements concerning either the heart or cardiac nerves in Fedele's papers. Subsequently, however, Florey (1966, page 215) felt that tunicate (*i.e.*, *Ciona*) hearts lacked "nervous control." Bone and Whitear (1958) described a plexus of nerves in the wall of the pericardium of *Ciona*, and stated the nerve fibers ran along the length of the heart. They suggested that these nerves were sensory. Apparently they did not see any branches of the nerve to the heart wall itself. Markham (1958) also observed cells evenly scattered along the suture of the *Ciona* heart which stained with methylene blue. He presumed these cells to be sensory. Kriebel (1968c) found the *Ciona* heart to have cholinoceptive properties, and he felt (1968a, page 450) that there could be an intrinsic regulation of the heart beat frequency. He maintained (1968a), however, that removal of either the raphe or the undifferentiated line from the isolated *Ciona* heart did not prevent heart reversals.

While Kriebel (1968a) found that some exposed-but-*in-situ* hearts of *Ciona* could beat for 8 hours without reversing, this was never observed in *Ecteinascidia* in any type of preparation (intact or experimental). Although Kriebel reported that body wall stimulation in *Ciona* increased the number of heart beats and decreased the reversal rates, this could not be statistically demonstrated in *Ecteinascidia*. Perhaps the level of external stimulation was not the same; the level was not precisely regulated in *Ecteinascidia*. According to Skramlik (1938) and Bacq (1934), the anterior (branchial) center of the intact *Ciona* heart has a greater frequency than the visceral center; according to Kriebel (1968a), this difference is evident at the beginning but not at the end of a pulsation series in isolated hearts. In *Ecteinascidia*, however, no statistically significant differences in total number of beats in a given cycle or their duration from either pole could be demonstrated in intact animals. Nevertheless, there is a tendency for the beats to be faster at the beginning than near the end of a given cycle in *Ecteinascidia* as in *Ciona*. In *Ciona* the heart rate is said by Kriebel (1968a) to depend upon the size of the tunicate. This could not be shown to be true for *Ecteinascidia*.

The heart of *Ecteinascidia* appears to differ in a number of other ways from that of *Ciona*. Thus, the *Ecteinascidia* heart does not swell at all after the pericardium is punctured, whereas in *Ciona*, the heart swells and fills the entire pericardial space (Kriebel, 1968a). While Kriebel (1968a) stated that he was able to abolish arrhythmic heart activity in intact *Ciona* by stimulating the animals to contract, erratic heart activity in *Ecteinascidia* was never thus abolished; in fact, causing them to contract often led to prolonged arrhythmia.

While Kriebel (1968a) found that 70% of the hearts of *Ciona* contracted irregularly when first isolated, 99.9% of the hearts of *Ecteinascidia* stopped beating altogether when first isolated. While 25% of the isolated hearts of *Ciona* would continue rhythmical beating after being cut in half (Kriebel, 1968a), none of the isolated hearts of *Ecteinascidia* beat rhythmically when so treated. In *Ciona*, Kriebel (1968a) found that collapsed and overdistended hearts could beat in only one direction; this phenomenon was never observed in *Ecteinascidia*.

There is no doubt that pressure directly over the heart of intact *Ecteinascidia* significantly increases the number of reversals. In *Ecteinascidia* this is probably effected via the nerve network. Although external pressure over the heart does lead to an increase in reversals, this does not mean that back pressure within the system can account for reversals as Florey (1966) proposed for *Ciona*. Thus, strong pressure on intact *Ecteinascidia* some distance away from its heart and the sudden release from this pressure did *not* elicit specific heart reversals. Also, the sudden loss of a massive amount of hemolymph, when the siphons were cut off, did not alter the reversal rate. Furthermore, strong continuous pressure directly over an inactive pacemaker did not prevent beats from originating within it.

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SUMMARY

1. The non-chambered tubular heart of the colonial ascidian *Ecteinascidia turbinata* is made up of a single layer of very long, thin, cross-striated and spirally-wrapped muscles. Enclosed within a large non-contractile pericardial sac, the heart and pericardium are innervated by a fine non-ganglionated network. The channels and vessels through which hemolymph circulates are non-contractile.

2. In recently captured, intact, and healthy-looking tunicates over a 5 year period, the heart exhibited either prolonged diastolic arrest, prolonged highly erratic (arrhythmic) beating, or fairly rhythmical but highly variable beating with periodic reversals in beat direction. No significant differences were found in contraction rates originating from either pole of the heart.

3. The heart begins to beat and reverse in the embryo and reversals continue throughout life. Synchronous reversals were not observed either between embryos from the same mother or between two or more attached individuals.

4. The extremely variable character of rhythmical heart beating involves both the number and duration of complete advisceral and abvisceral cycles. The variability is not due to (a) developmental stage (embryo, tadpole, mature individuals), (b) body length, (c) time in sea table after captivity, or (d) amount of sea water in examination chamber.

5. The great variability of heart rhythmicity in intact animals diminishes markedly within the first hour after isolation, if the tunicates are left undisturbed. The heart is capable of beating and reversing with remarkable uniformity for long periods both in unchanged and in constantly changing sea water in small chambers. Simple manipulation of intact tunicates can readily and quickly induce renewed and extreme cardiac variability.

6. Pressing externally directly over the anterior (advisceral) pole of the heart or over the center of the heart shortly after the beginning of either the advisceral or abvisceral cycle significantly increases the rate of heart reversal.

7. Hearts continue to beat and reverse for a long time after both siphons (including the "brains") are removed. When the pericardium alone of the heart itself is punctured with a fine needle or cut with scissors, the heart always stops instantly in diastole for variable periods of time. The two halves of a centrally-severed heart are capable of reversing. Heart reversals were observed after puncturing small portions of the anterior and posterior poles of the heart. When the anterior pole was completely ablated, heart beats were observed only from the posterior (abvisceral) pole. Semi-isolated hearts were never observed to beat normally and rhythmically.

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THE EFFECTS OF LIGHT AND TEMPERATURE ON ATP LEVEL AS A MEANS OF DETERMINING AGGREGATION IN THE CELLULAR SLIME MOLDS

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Morphogenesis in the Acrasiales is determinable both by temperature and by light. Phototropism was first reported in *Dictyostelium mucoroides* by Potts (1902) and by Olive (1902). These authors observed that developing fruiting structures were positively phototropic and that smaller fruiting bodies developed in light than in darkness. Similar observations have been made by Harper (1932) and by Raper (1940) for *Polysphondylium violaceum* and for *Dictyostelium discoideum* respectively. Raper (1940) moreover noted that migrating pseudoplasmodia of the latter species were singularly sensitive to light, and Bonner, Clarke, Neely and Slifkin (1950) showed the anterior end to be the most photosensitive. More recently, Olive and Stoianovitch (1960) have reported that the amoebae of *Acrasis rosea* will not develop beyond the vegetative stage, and will merely encyst if deprived of light; whereas under full illumination, full development will take place.

That cold might have an effect in delaying aggregation and morphogenesis was first demonstrated by Potts (1902) and later confirmed by Raper (1940) who found that elevation of temperature accelerated aggregation and morphogenesis, whereas cold had the opposite effect. More recently Konijn (1965) has further demonstrated the delaying effect of cold, and he and Raper (Konijn and Raper, 1965) and also Kahn (1964) have confirmed the earlier observations on light. This has shown a complex interrelationship between time of photoperiod and development that is reminiscent of the interactions prevailing in higher plants.

The process of aggregation in the cellular slime mold involves a transition of the myxamoebae from a nonadhesive to an adhesive stage. The parameters of adhesion are therefore highly relevant to the processes of aggregation and of morphogenesis.

Recently the author proposed a contractile protein model for cell adhesion based on the properties of actomyosin-like proteins (Jones, 1966). In this model, a high intra- (or extra-) cellular ATP level would initiate the contraction of surface or subsurface elements, resulting in an increase in surface charge density, which on the basis of the Verwey-Overbeek (Verwey and Overbeek, 1945) theory of the stability of lyophobic colloids, would result in impaired adhesion. Conversely, elevation of intracellular (or extracellular) ADP would (probably by product inhibition) allow the expansion of the protein, thus reducing surface charge density and permitting adhesion.

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[1] Dr. P. C. T. Jones died on February 15, 1971 during the review process of this paper. He was a Lecturer in Zoology at Aberystwyth and was to be promoted to Senior Lecturer this year. Final revision of this manuscript was then carried out by his colleague Dr. I. ap Gwynn, Research Associate in the Cell Research Unit at Aberystwyth, and proofs marked in the editorial office.

On this basis, therefore, it would be expected that a low intracellular ATP level would favor aggregation whereas a high level would prevent this. Indeed, Woolley and Jones (1970) have shown that the ATP level and ATP/ADP ratio of free myxamoebae are higher than those of their adhesive counterparts. The present author, moreover, has shown (Jones, 1969a, 1969b, 1970a, 1970c) that ascites tumor cells, chick embryos and plant tissues respond to cold by elevation of their intracellular ATP levels, which are however diminished in darkness.

Indications that such an effect is demonstrable in *Dictyostelium discoideum* have recently been obtained by the author (Jones, 1970b). It might, therefore, be expected that light and cold would have opposing effects, not only in determining intracellular ATP levels but also the timing of aggregation and of morphogenesis, in cellular slime molds.

A more extensive study was therefore made to determine whether a direct correlation could be established between the influence of light and cold in determining the timing of aggregative and morphogenetic events in the life history of both *Dictyostelium mucoroides* and *Polysphondylium violaceum* and their influence on intracellular ATP levels and ATP/ADP ratios.

Materials and Methods

Myxamoebae of *Dictlyostelium mucoroides* (strain IMI 74707b from the Imperial Mycological Institute, Kew, England) and of *Polysphondylium violaceum* (strain 217.57 from the Centraalbureau voor Schimmelcultures, Delft, Holland) were grown in mixed culture on nutrient agar (de Haan, 1959) with the bacterium *Aerobacter aerogenes* (16c). The amoebae were harvested when nearly all the bacteria had been consumed. The harvested amoebae were then washed with cold glass distilled water, and separated by spinning at 250 g for 10 minutes at 4° C. The procedure was repeated four times to free the amoebae from bacteria, after which the packed amoebae were resuspended in twice their volume of buffered standard solution (Bonner, 1947) at pH 7.2.

The amoebae suspension was then spread on the surface of 2.2% agar (buffered to pH 7.0 with 10 mM tris) in rectangular (10″ × 12″) perspex dishes. These were then exposed to dark or to 40 lumens per sq ft cold white fluorescent light at either 10° or 20° C. At the end of four hours, the amoebae were harvested with a glass scraper (microscope slide) and dropped directly into liquid nitrogen in a chilled mortar. After grinding to a fine powder, this was then triturated firstly with 2 ml 0.6 M perchloric acid and then with 2 ml 0.3 M perchloric acid (at 4° C). On thawing, the mixture was kept at 4° C and, after homogenizing in a modified Potter homogenizer with a "Telflon" pestle, made up to 10 ml with double glass distilled water in a graduated centrifuge tube. After spinning at 2500 rpm for 5 minutes to remove denatured protein and cellular debris, the supernatant fluid was then neutralized with drops of saturated K_2CO_3, filtered after 5 minutes, and then kept at 4° C prior to nucleoside phosphate estimation.

ATP was then estimated by a standard method (Adam, 1963) using an UV-test kit based on phosphoglycerate kinase (Boehringer und Sohne, Mannheim, Germany). As it has been shown previously (Woolley and Jones, 1970) that ATP was the principal nucleoside triphosphate present in *Dictyostelium* myxamoebae, this method was used despite its inability fully to differentiate ATP from GTP, CTP and UTP, on account of the ready availability of the necessary enzymes and reagents in kit form.

In this test, ATP is estimated by spectrophotometry, using a method based on the oxidation of DPNH as in the following reactions:

(1) 3:phosphoglycerate + ATP = 1:3:diphosphoglycerate + ADP

(2) 1:3:diphosphoglycerate + DPNH + H^+
= 3:phosphoglyceraldehyde + PO_4^- + DPN^+

ATP was therefore estimated by difference in optical density at 340 or 366 $m\mu$ by spectrophotometry using an Unicam SP 500 spectrophotometer. Detailed procedure is as described elsewhere (Jones, 1969a, 1969b, 1970a). When ADP and AMP were also estimated, an UV-kit was similarly used (Adam, 1963) (Boehringer und Sohne, Mannheim).

The centrifuge pellet obtained after perchloric acid extraction of the myxamoebae was weighed after drying; and subsequently dissolved in 7.5% NaOH for protein estimation by the Biuret reaction.

To 3 ml of a prepared Biuret reagent (Schweizerhall, Basle) were added 0.2 ml of the solubilized pellet; and the optical density at 540 $m\mu$ read after 30 minutes and compared with a blank. The protein present was then calculated by reference to a standard curve prepared by the use of serum albumin.

Nucleoside phosphate levels were then calculated on the basis both of protein and of dry weight. A check on the efficacy of the estimation procedure was made by adding 0.2 ml 5×10^{-4} M ATP to a sample cuvette, when recoveries of the order of 90% were obtained.

Time of aggregation was determined by the method of Konijn and Raper (1965). In this method, a suspension of myxamoebae as prepared above is reconstituted and adjusted to 4×10^5 cells/ml. One hundred μl drops of this are deposited on non-nutrient agar in dim fluorescent light.

Populations at a density of about 100 myxamoebae/mm^2 were inoculated into Petri dishes which were left uncovered until excess surface water had disappeared.

These were then incubated in either cold white fluorescent light (430 lux) or dark at either 13° C or 23° C. Replicate dishes were examined and sacrificed at half-hourly intervals in order to ascertain the onset of aggregation. This was determined as the average time when streaming to a center could be clearly defined. The results represent the means of three replicate experiments in each case.

RESULTS

The results (Table I) indicate that with both *Dictyostelium mucoroides* and with *Polysphondylium violaceum* the effect of light is to reduce ATP level and ATP/ADP and ATP/AMP ratios. On the other hand reducing temperature has the opposite effect and all these values are raised.

Moreover, it will be seen in Table II that in both species studied, the time of aggregation was delayed by cold and advanced by light. Furthermore, an

Table 1

The effect of cold white fluorescent light and of temperature on ATP level, ATP/ADP and ATP/AMP in the myxamoebae of Polysphondylium violaceum *and on ATP level in* Dictyostelium mucoroides

(A.) *Polysphondylium violaceum*

		13° C	23° C
	ATP level (mμ Moles/mg protein)		
Light (430 lux)		9.45 ± 0.92	2.94 ± 0.72
Dark		12.49 ± 1.89	9.53 ± 1.01
	ATP/ADP		
Light (430 lux)		4.50	1.40
Dark		5.81	3.50
	ATP/AMP		
Light (430 lux)		5.39	1.27
Dark		8.00	3.60

(B.) *Dictyostelium mucoroides*

	ATP level (mμ Moles/mg protein)		
Light (430 lux)		5.78 ± 0.79	4.17 ± 0.62
Dark		17.81 ± 3.14	10.35 ± 2.06

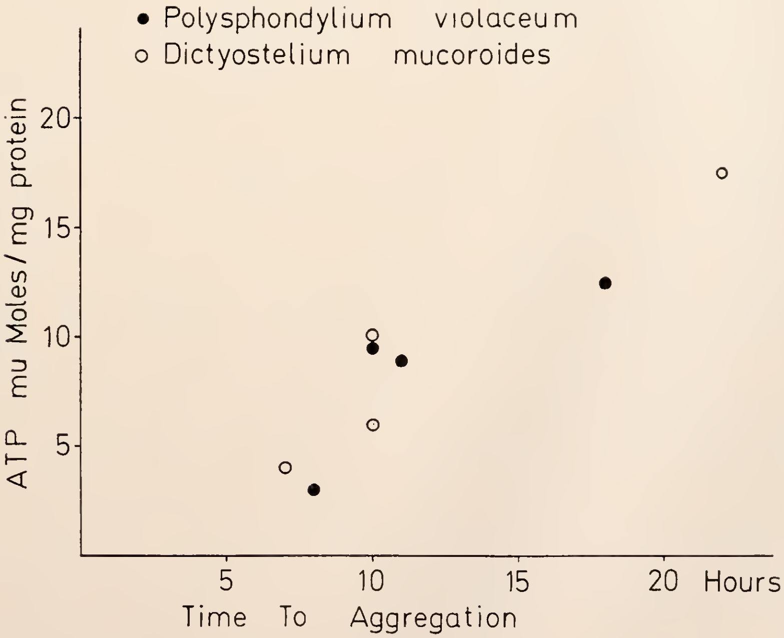

Figure 1. Graph of ATP level, as determined by the method of Adam (1963), plotted as an ordinate against the time of aggregation in hours for *Polysphondylium violaceum* and *Dictyostelium mucoroides*.

interesting correlation between time of aggregation and ATP level is discernible. In Figure 1, ATP level is plotted as an ordinate against time of aggregation. It is immediately apparent that a direct relationship between these two parameters for the species studied and this result supports the author's proposition that the level of ATP and of other nucleoside phosphates is an important determinant of cell adhesion and aggregation (Jones, 1966; Woolley and Jones, 1970).

DISCUSSION

A number of hypotheses of greater or lesser complexity have been advanced to explain the effects of light and of temperature in determining aggregation and morphogenesis in the cellular slime molds.

It has been proposed, for instance, that light may act in some way by suppressing a center-suppressing substance, and Bonner and Hoffman (1963) have suggested that this may be CO_2. Indeed these authors have described both gas-sensitive and gas-insensitive species. Again, it has been suggested that light may exert its influence by heating the myxamoebae or pseudoplasmodia and thus invoke a temperature effect. Indeed Bonner, Clarke, Keely and Slifkin (1950), in demonstrating that slugs of *Dictyostelium discoideum* oriented to both light and heat, have shown that quite small heat gradients (a difference of as little as 0.05° C/cm exists between the anterior and posterior end of an aligned amoeba) are involved, and they suggest that light may exert its influence by differential heating. Whatever the mechanism, the present results indicate a positive correlation between ATP level or ATP/ADP ratio and the time of aggregation in cellular slime molds. Indeed a wide range of developmental processes may be determined in this manner (Jones, 1969b). For instance, the author has demonstrated that cold and light counter each other's effect in determining ATP levels in the cells of higher plants, and has suggested that his may be the means whereby the interactions of light and of cold in the photoperiodic response, and in breaking of dormancy, may be determined (Jones, 1970a). A similar effect is demonstrable in the whole chick embryo where reduced ATP level can be correlated with photo-acceleration of development (Jones, 1970c). On this basis therefore, it would be expected that a whole sequence of events would result from light- and

TABLE II

The effect of cold white fluorescent light of temperature on the time of aggregation of myxamoebae of Polysphondylium violaceum *and* Dictyostelium discoideum

Average time of aggregation (hours)		
(A.) *Polysphondylium violaceum*		
	13° C	23° C
Light (430 lux)	11	8
Dark	18	10
(B.) *Dictyostelium discoideum*		
Light	10	7
Dark	22	10

cold-induced alterations in ATP level. Both adhesiveness and protein synthesis (Naguib and Christopherson, 1965) would probably be affected, and it is not difficult to see how a developmental course of events might be determined. The effect of light on the aggregative movements of myxamoebae and of pseudoplasmodia is also worthy of consideration. In *Amoebae proteus,* Mast (1940) suggested that response to light was by gelation of the cytoplasm of the illuminated pseudopod. This, on the basis of the present observations is consistent with diminution of ATP and increase in ADP and AMP, and concomitant with increased cellular adhesiveness (Jones, 1966; Woolley and Jones, 1970). There is moreover no reason to suppose that the small "limax" amoebae of the Dictyosteliasceae should behave in a manner different to that of their large "proteus" counterparts, and it is therefore probable that the altered aggregative behavior of the former derives from light induced diminution of ATP level.

It is also probable that levels of cyclic AMP as an "acrasin" (Konijn, Barkley, Chang and Bonner, 1968) are related to those of other nuceloside phosphates, which may each have different functions, the former as an attractant and the later as mediators of cellular adhesiveness (Jones, 1966; Woolley and Jones, 1970).

Such considerations are of even greater interest from the standpoint of the theory of amoeboid movement proposed by Goldacre and Lorch (1950). In this theory, the gelated peripheral cytoplasm is solated in the tail, and then propelled forward subsequently to gelate in the formation of pseudopods. Goldacre suggested that the amoeba thus made use of the free energy of ATP to solate cytoplasm in the tail, this energy being subsequently used for osmotic and mechanical work in amoeboid movement. Evidence for this theory was adduced on the basis of microinjection of ATP which solated the amoeboid cytoplasm, and could also be used to induce "tail" formation.

The present author extended certain aspects of this work in his model for cell adhesion (Jones, 1966) by observation of the amoeba "tail" when challenged with ATP or ADP. It was found that whereas ADP caused movement to cease, presumably by "tail" expansion, activity could be reinstated by subsequent application of ATP. Moreover, it was found that the ATP treated amoeba had less purchase of the substratum than had the ADP treated cell, and was thus less adhesive. A similar effect can be demonstrated with the myxamoebae of *Dictyostelium discoideum.*

Microinjection of ATP reduces cytoplasmic viscosity in amoebae (Goldacre and Lorch, 1950). As will be seen above, the effect of lowering temperature or decreasing illumination is to increase ATP level. That this can be correlated directly with a concomitant decrease in cytoplasmic viscosity (Heilbrunn, 1956) is therefore not surprising. This effect may result from a direct inactivation of an ATPase by cold (Penefsky and Warner, 1965) or possibly by a direct effect on an intracellular thermodynamic equilibrium involving actin and myosinlike proteins and ATP (Jones, 1969a). On the basis of the author's model for adhesion, chilled amoebae would be expected to be non-adhesive and mobile; and not readily aggregate. On the other hand, under conditions of high illumination, ATP level is reduced, and by the arguments presented above, the myxamoebae would become more adhesive and less mobile, and would thus more readily aggregate. Moreover a reason for the local gelation observed by Mast in *Amoeba proteus* pseudopodia becomes evident for it may derive from a local diminution of ATP level, although it is difficult to disentangle cause and effect.

It is therefore not difficult to see how light and temperature might engage in a complex interaction in determining aggregation and morphogenesis in the cellular slime molds. The possible effect of CO_2 mentioned above (Bonner and Hoffman, 1963) may be mediated in this manner also, for it may well affect respiratory and glycolytic processes with a consequent diminution in ATP level.

It must be emphasized that, although in general in the Acrasiales, light accelerates and cold delays development; there are minor departures from this generalization. Konijn and Raper (1965) for instance have shown that with *Dictlyostelium discoideum* NC-4(H) optimal aggregation occurs with an initial dark period of 7–9 hours followed by 4–2 hours light. Similar results have also been obtained with some other strains of Dictyostelium discoideum (Konijn and Raper, 1965), whereas those previously used by the author (Jones, 1970b) (*Dictyostelium discoideum* Acr. 12) needed constant light for optimal aggregation. This was also the case with both *Dictyostelium mucoroides* and *Polysphondylium violaceum* in the present study.

Konijn, Barkley, Chang and Bonner (1968) have moreover recently identified cyclic AMP as an "acrasin." Should this be further substantiated, then it might well be expected that alterations of cyclic AMP level would be concomitant with the changes in other adenosine nucleotides as seen in the results presented above.

It is interesting to note that photoacceleration of development is not restricted to the Acrasiales. Such an effect is readily demonstrable in higher plants and in the development of the chick, where Lauber and Schutz (1964) have demonstrated the acceleration of development following illumination of eggs. Moreover, that this effect was due to light energy alone was determined by assessment of the temperatures of the air sacs, which were found to be sensibly constant. The above authors came to the conclusion that light mediated its effect over the whole embryo, and was not limited to an optic response. The present author's observations (Jones, 1970c) that embryos in illuminated eggs have lower ATP levels than those incubated in the dark is consistent with the observations of the present paper and suggests that a central effect on cell metabolism through ATP level (and possibly that of other related metabolites) is the transducing mechanism of parameters of state such as temperature, pressure and light.

In this manner it may well be that not only are morphogenetic events determined, but also the synchronization of biological clocks. Bünning (1967) states that cycles of high and low temperature regulate in such a way that the phase of low temperature coincides with the physiological state reached during the night. The results presented above give an explanation of this common effect, and suggest that variations in ATP level are the central feature of such rhythms.

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RESPIRATORY REGULATION IN *BUFO ARENARUM* EGGS [1]

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The study of the respiratory response of whole homogenates and the use of 2,4-dinitrophenol (DNP) as an uncoupling agent of oxidative phosphorylation, have proven to be useful tools to shed some light on the problem of respiratory control during amphibian development. The well established fact that an enhancement of respiratory activity can be obtained by homogenization (Spiegelman and Steinbach, 1945; Gregg and Ray, 1957; Gregg, 1960) as well as by treatment with DNP (Gregg, 1960; Legname, 1968), indicates that the *respiratory potential* exceeds the *respiratory norm, i.e.,* the respiratory rate exhibited by intact embryos under normal conditions (Gregg, 1960). That is to say that the developing egg relies on a respiratory system which is more than adequate to support the rate of normal respiration.

No conclusive evidence is available, however, regarding the mechanism by which the respiratory norm, held below the respiratory potential, increases as a function of developmental age. This was explained by Spielgelman and Steinbach (1945) and also by Gregg and Ray (1957), as a consequence of structural changes occurring in the course of development, which allow previously separated enzymes to contact their substrates. Later on, Gregg (1960) assumed that increasing respiratory rates would be the result of a parallel increase in the rate at which ATP is metabolized in response to energy requirements. Thus, as has been found in other cell systems, any decrease of the ATP pool with a concurrent increase of ADP and inorganic phosphorous, would activate oxygen consumption until the ATP level is re-established.

However, some unexpected findings have also been reported. For example, the homogenates of *Rana pipiens* eggs, prepared before the onset of gastrulation, were found to respire at about the same rate as intact eggs, in contrast to the respiration response observed following homogenization of older embryos (Gregg, 1960). Further, Gregg (1960), in the same species, and Legname (1968), in *Bufo arenarum,* failed to raise the respiratory activity of egg homogenates by means of DNP, in contrast to the marked effect obtained by treatment of intact eggs.

Two hypotheses have been proposed by Gregg (1960) to account for these observations. The failure of homogenization to elevate the respiratory activity of the segmenting egg has been ascribed to the formation of some coat, probably of lipo-protein nature, around the respiratory particulates impairing the availability of respiratory substrates. As to the lack of effect of DNP on egg homogenates, Gregg (1960) suggests that it could be the result of an activation of ATPase following cell disruption. Since ADP and inorganic phosphorus, under these conditions, have already reached a maximum level, no DNP effect can be expected.

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The present study was undertaken as an experimental approach to investigate the mechanisms underlying these puzzling effects with special reference to the question of embryonic respiratory control.

MATERIAL AND METHODS

Bufo arenarum oocytes were obtained by injecting adult females with a suspension of homologous hypophysis preserved according to Pisanó (1956). Fertilization was performed *in vitro* and embryos were reared in 10% amphibian Ringer solution without bicarbonate at laboratory temperature. The jelly coat was eliminated with 2% thioglycollic acid neutralized with KOH, followed by repeated washings with Ringer solution. Embryonic ages were determined by using the chart of Del Conte and Sirlin (1951) for the development of this species.

Homogenization was performed in a glass homogenizer with a teflon pestle, operated in an up-down manner by hand. The degree of homogenization was determined by the number of strokes which, except otherwise stated, was always six. Suspension media are indicated in each step. Homogenization was carried out in an ice cold bath.

The oxygen uptake was determined by the direct Warburg method using 17 ml reaction flasks with sidearm and center wells. Carbon dioxide was absorbed with 20% KOH contained in the center wells. The flasks were shaken at 80 cycles per minute at an ampltidue of 7 cm. Readings were made at 15 minute intervals.

When necessary, the following concentrations of cofactors and substrate were added to the homogenates: 0.12 μM Cytochrome C, 1.3 μM nicotinamide adenine dinucleotide (NAD) and 25 μM potasium fumarate. Final concentration of 2,4-dinitrophenol was 1.5×10^{-4} M. The final volume of the system was always 3.0 ml.

In order to determine ATPase activity, aliquots of the homogenates were incubated according to Maruyama and Ishida (1954), using 0.15 M histidine-KCl buffer, instead of sucrose. The liberated inorganic phosphorous was evaluated according to Marsh's technique (1959), microadapted to estimate 0.1 gamma (Puszkin, 1969).

RESULTS

Respiratory effects of homogenization

Since Spiegelman and Steinbach (1945) and Gregg (1960), have reported contradictory data regarding the effects of homogenization on the respiration of *Rana pipiens* pregastrular embryos, experiments have been carried out in order to clarify inconsistencies.

The suspension media used by the above mentioned authors, 0.66 M phosphate buffer and 0.01 M phosphate buffer in 0.065% NaCl, respectively, have been tested. Under our working conditions, the suspension media do not affect significantly the respiratory relation of homogenized (as compared to intact) eggs. Homogenated respiration remained below that of intact controls (Table I).

TABLE I

Respiration of homogenized and intact eggs in different salt solutions. [*Values are expressed as μl of oxygen taken up; intact eggs: 300 blastulae per flask; homogenates: 3 ml containing 300 blastulae (100 blastulae per ml).*]

Eggs	0.066 M phosphate buffer		0.01 M phosphate buffer in 0.065 % NaCl	
	15 min	30 min	15 min	30 min
Intact	6, 3	14, 1	6, 1	13, 8
Honogenized	3, 1	6, 5	3, 5	6, 0

The number of eggs is another factor which has also been considered. Spiegelman and Steinbach (1945) used concentrations calculated to obtain 5 mm readings with 10 minute intervals, while Gregg uses 50 eggs per flask. Under our working conditions, such concentrations would correspond to 400–500, and 100–150 blastulae per flask, respectively. Table II shows that the respiratory ratio between intact and homogenized eggs varies according to the number of embryos used. In fact, while 150 homogenized blastulae do not exhibit respiratory activity, the oxygen uptake determined by 300 homogenized blastulae is equivalent to about 50% of that registered for the same number of intact eggs. Values *above* intact controls were obtained when using 500 homogenized blastulae.

When similar experiments were carried out adding cofactors and a substrate of the tricarboxylic acid cycle to the medium (Table III), the respiration of homogenates always exceeded that of intact eggs, regardless of the number of embryos used.

Respiratory effects of DNP on the homogenates

The lack of respiratory effects of DNP on homogenized amphibian embryos reported by Gregg (1960) in *Rana pipiens* and by Legname (1968) in *Bufo arenarum* has been reviewed. DNP respiratory effect depends upon the mechanical treatment to which cells have been submitted during brei preparation. In fact, Table IV shows that when working with early cleavage stages, which exhibit a

TABLE II

Effect of the egg number on the respiration of homogenates. [*Values are expressed as μl of oxygen taken up by 3 ml of different concentration of homogenized blastulae; suspension medium: 0.1 M phosphate buffer at pH 7.0; intact eggs: 150, 300 and 450 blastulae per flask; final volume: 3 ml.*]

Number of eggs per ml	15 minutes		30 minutes	
	Intact	Homogenized	Intact	Homogenized
50	3.8	0.0	7.6	0.0
100	6.3	3.0	12.6	4.6
150	9.0	14.6	20.1	29.3

TABLE III

Respiration of homogenized eggs in the presence of exogenous sustrate and cofactors. [*Values are expressed as μl of oxygen taken up by 3 ml of different concentration of homogenized blastulae supplemented with cytochrome C, NAD and postasium fumarate; suspension medium: 0.1* M *phosphate buffer at pH 7.0; intact eggs: 150, 300 and 500 blastulae per flask; final volume: 3 ml.*]

Number of eggs per ml	15 minutes		30 minutes	
	Intact	Homogenized	Intact	Homogenized
50	3.0	6.6	5.6	13.2
100	6.1	13.1	13.8	27.5
167	9.0	21.5	21.1	46.4

considerable cell size, and using a dense suspension medium, it is possible to regulate homogenization so as to obtain a cell-free medium that can be stimulated by DNP, while a more drastic homogenization increases the oxygen uptake, and eliminates the effect of the uncoupling agent. Similarly, on very gently homogenized uncleaved eggs, DNP determines a respiratory increment similar to that detected on intact eggs (Table V).

TABLE IV

Influence of the degree of homogenization on the respiration of homogenates in the presence of 2,4-dinitrophenol. [*Values are expressed as μl of oxygen taken up by 3 ml of homogenized first cleavage eggs containing 167 eggs per ml, suspended in a very dense medium* (*sucrose 1.2* M); *final pH 7.0*]

Degree of homogenization (pestle strokes)	15 minutes			30 minutes		
	Control	DNP	Δ%	Control	DNP	Δ%
2	8.2	15.0	82	18.0	32.1	76
10	14.7	14.7	0.0	32.0	30.0	−6

TABLE V

Respiratory effects of 2,4-dinitrophenol on homogenized and intact eggs. [*Values are expressed as μl of oxygen taken up by 3 ml of homogenized uncleaved eggs containing 167 eggs per ml, suspended in a dense medium* (*sucrose 1.2* M); *final pH 7.0, degree of homogenization: One pestle stroke; intact: 500 uncleaved eggs per flask in 10% Ringer's solution, final volume 3 ml.*]

Eggs	15 minutes			30 minutes		
	Control	DNP	Δ%	Control	DNP	Δ%
Intact	7.6	39.5	420	14.8	66.6	463
Homogenized	6.0	31.2	420	18.0	55.0	206

Mechanisms involved in the respiratory increment

In order to explain the mechanisms involved in the respiratory increment determined by homogenization, Gregg's hypothesis ascribing this effect to the stimulation of ovular ATPases resulting from cell disruption has been evaluated.

For this purpose, aliquots were taken from egg batches homogenized with variable intensity, in order to determine the oxygen uptake and the liberated inorganic phosphorous. Table VI shows that both oxygen consumption and ATPase activity, expressed as liberated inorganic phosphorus, increase with a marked parallelism according to the degree of homogenization.

TABLE VI

Effect of homogenization on oxygen uptake and ATPase activity. [Oxygen taken up and inorganic phosphorus liberated by 3 ml of homogenized first cleavage eggs (100 eggs per ml). Homogenates were supplement with citochrome C, NAD and potassium fumarate; suspension media: Histidine-KCl buffer at pH 7.0.]

Degree of homogenization (pestle strokes)	Oxygen (µl)		P_i (µM)
	15 min	30 min	
1	6.6	13.2	0.33
3	9.0	18.1	0.45
6	11.0	21.1	0.57

On the other hand, Table VII shows that the respiratory increment determined by homogenization disappears after the addition of an ATPase inhibitor such as sodium fluoride (NaF). This same table demonstrates that the respiration of homogenates, already above that of intact controls, can be further increased by adding ADP to the medium. The addition of ATP, on the contrary, does not affect the respiration of homogenates treated or not with NaF.

TABLE VII

Effects of NaF, ADP and ATP on the respiration of homogenized eggs. [Values are expressed as µl of oxygen taken up by 3 ml of homogenized blastulae (100 eggs per ml) supplemented with citochrome C, NAD and potassium fumarate; ATP and ADP added: 50 µM; NaF: final concentration, 0.04 M; intacts: 300 blastulae per flask, final volume: 3 ml, suspension media: 0.1 M phosphate buffer at pH 7.0.]

Minutes	Intact	Homogenized				
		Control	NaF	ADP	ATP	ATP + NaF
15	4.6	10.4	4.5	18.5	10.9	4.9
30	8.3	17.1	9.1	33.4	18.9	9.8

Discussion

The results described in the above section show that the effect of homogenization on the respiration of pregastrular embryos is independent of the suspension medium used, while the concentration of embryonic material appears to be of utmost significance in the relative rates of respiration of homogenized, as compared to intact, eggs.

These results also suggest that the dilution of the egg material, or of some of its components, would prevent the manifestation of a respiratory increment determined by homogenization. In this connection our results show that the respiration of homogenates may be made to duplicate that of the intact controls by adding cofactors as NAD and cytochrome C, and fumarate as a substrate, independently of the number of eggs used. In agreement with these results, isolated mitochondria of *Bufo arenarum* blastulae have been found to oxidize fumarate at a very high rate (Salomón de Legname, 1969).

The contradictory results obtained by Spiegelman and Steinbach and by Gregg, could be ascribed to the different concentrations of embryos used by these authors. The lack of effect reported by Gregg (1960) could be the consequence of a dilution of substrates and electron carriers, not occurring in Spiegelmann and Steinbach's experiences, which were performed using high concentrations of biological material.

As to the mechanisms determining an increment of oxidations by homogenization, Gregg (1960) also suggests that cell breakage may stimulate ATPase activity, thus resulting in an increase of ADP and inorganic phosphorus levels and, consequently, in an increment of respiration. Our results support this assumption (since a more drastic homogenization provokes a proportional increment on the respiratory and ATPase activity), and demonstrate in agreement with Gregg's hypothesis, that cell disruption determines an increment in the oxygen uptake through ATP hydrolysis.

On the other hand, the present results concerning the effects of concentration of embryos suggest an alternative to Gregg's hypothesis, which ascribed the failure of homogenization to increased respiration of very young embryos, to the formation of a membrane that would limit respiration to the capacity of certain substrates to penetrate lipoprotein barriers.

Confirmation of the present hypotheses could also account for the lack of respiratory effects of DNP in homogenized embryos reported by Gregg (1960) in *Rana pipiens* and by Legname (1968) in *Bufo arenarum*. In fact, since the activation of ovular ATPases seems to depend upon the mechanical treatment to which cells are submitted during brei preparations, the use of a very gentle homogenization which would not stimulate ATPases significantly would permit the action of the uncoupling agent. Such effect would disappear with a more severe homogenization. This hypothesis has been confirmed by using a very dense suspension medium and early-cleaving embryos, which exhibit a considerable cell size and permit a very gentle homogenization. Working with very carefully homogenized one-cell eggs, DNP determines a respiratory increment similar to that registered in unbroken controls.

Both factors, homogenization and DNP, stimulate oxygen uptake by altering the ATP:ADP ratio; therefore, the control of respiration during embryogenesis is likely to depend upon a similar mechanism in which ATPases would be most importantly involved.

The use of 0.04 M NaF as an ATPase inhibitor, show that the embryonic respiratory increment depends mostly on the concentration of ADP in the medium, while the role of ATP during the respiratory processes would be limited to that of an ADP donor. The slight increment in respiration detected after the addition of ATP, could be ascribed to the small amounts of ADP contaminating this compound.

Although NaF is not a specific ATPase inhibitor, no effect other than that can be ascribed to this compound under our working conditions. Its action would be limited to the annulment of the respiratory increment resulting from homogenization, while the oxygen uptake of intact eggs remain unaffected. In this connection, Barbieri and Valdez Toledo (1966) although working with lower concentrations, demonstrated that the respiration of *Bufo arenarum* blastulae is not affected by NaF. This effect had already been reported by Barth and Barth (1954) for *Rana pipiens.*

This assumption is further supported by the fact that NaF is known to act mainly at the level of the Embden-Meyerhof pathway, which accounts for only 20% carbohydrate degradation in *Bufo arenarum* blastulae. The remaining 80% is broken down via the pentose phosphate cycle (Salomón de Legname, Sanchez Riera and Sanchez, in preparation).

SUMMARY

The more plausible hypotheses regarding the mechanism which controls amphibian embryonic respiration have been reviewed, providing some explanation for the contradictory respiratory ratios between intact and homogenized eggs reported by other investigators.

The concentration of embryonic material appears to be of the utmost significance in the relative rates of respiration of homogenized as compared to intact eggs. A close correspondence between increased oxygen uptake and the ATPase activity resulting from homogenization has been demonstrated. This would account for the lack of respiratory effect by 2,4-dinitrophenol which has been described in homogenized eggs of *Rana pipiens* and *Bufo arenarum.* Homogenization, as well as 2,4-dinitrophenol could stimulate oxygen uptake by altering the ATP/ADP ratio. By means of ATPase inhibition it has been also demonstrated that the respiration of homogenates is strongly affected by ADP while exogenous ATP is almost without effect.

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LARVAL DEVELOPMENT OF *PAGURUS LONGICARPUS* SAY REARED IN THE LABORATORY. IV. ASPECTS OF THE ECOLOGY OF THE MEGALOPA[1]

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Many animals are able to delay metamorphosis to the juvenile or adult stage until some stimulus has been received from a suitable substrate (Wilson, 1952, 1958; Crisp and Barnes, 1954; Scheltema, 1961; and many others). In some cases the physiological basis for delayed metamorphosis has been carefully investigated (for example, Meadows, 1964a, 1964b, 1964c).

During or immediately after the megalopal instar, the hermit crab must find a suitable shell in which to house its abdomen in order to protect itself from predation. For oceanic species, delayed metamorphosis might be an adaptation to permit larvae to reach the bottom while still capable of locomotion in the pelagic environment (Bouvier, 1905). Thompson (1903) suggested that lack of a shell could delay metamorphosis. Thompson (1903) and Bookhout (1964) have both reported that megalopae without shells have a higher per cent mortality than those with shells. If this is true, it implies physiological stress resulting from lack of a shell as does the observation that shell-less adults do not feed (Allee and Douglis, 1945).

None of these hypotheses has been rigorously tested in controlled experiments. Thompson (1903) tested the effect of various shells on megalopae, but his conclusions are questionable (see below). Reese (1962, 1968) and Hazlett and Provenzano (1965) have studied the behavior associated with selection and entry of shells by megalopae. Reese (1968) has made some observations on the role of shells in emigration of *Birgus latro* onto land and metamorphosis to the juvenile.

The present study was made specifically to test the effect of shell presence on mortality and intermolt duration of the megalopa of *Pagurus longicarpus*.

MATERIALS AND METHODS

Megalopae used in these experiments were obtained from a number of different cultures established for various experiments on zoeal instars. Culture conditions up to the megalopa differed between experiments, but were the same within each given experiment. In all experiments, the salinity was maintained as it was during zoeal development (23.7‰ in Experiment 1, 30.0‰ in Experiment 2, 25.5‰ in Experiment 3). Temperature was quite variable as culture dishes were kept on a sea table. The mean temperature was 24° C (21–26° C) in Experiment 1, 24.5° C (19–27° C) in Experiment 2, and 22° C (18–24° C) in Experiment 3. Megalopae were transferred to freshly prepared environments on a variable schedule. Mortality, molting, and shell entry were noted daily. The shells in every experiment were *Bittium*, cleaned of organic matter with KOH. These proved very suitable in size and were easily collected from *Zostera* beds where they abound on nearly every blade.

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In Experiment 1, three variables were involved: sand substrate, shell, and nauplii of *Artemia*. Eighteen megalopae were maintained individually in compartmented plastic boxes in each of six different environments, making a total of 108 megalopae for the entire experiment. The six environments were: no sand substrate, no shell, *Artemia;* no sand substrate, shell, *Artemia;* sand substrate, no shell, *Artemia;* sand substrate, shell, *Artemia;* sand substrate, no shell, no *Artemia*, sand substrate, shell, no *Artemia*.

In Experiments 2 and 3 the design was greatly simplified with only a single variable, shell presence or absence. No sand substrate was provided; nauplii of *Artemia* were added as food. In Experiment 2, 62 megalopae were presented a shell, 61 were not, for a total of 123 megalopae. In Experiment 3, 115 megalopae were presented a shell, 113 were not, for a total of 228. These experiments differed in the salinity-temperature regime to which the megalopae were subjected and hence are not exactly comparable.

RESULTS

Mortality

In Experiment 1, no significant difference in numbers alive and dead was detected at the 95% confidence level among the various treatments when tested by an R × C contingency test, indicating that there was no effect of any treatment on mortality. Per cent mortality for megalopae with shells was 16.7% compared to 13.0% for those without shells, 14.8% overall. Some deaths are known to have resulted from predation by small *Nereis* sp. introduced with the sand substrate (about 5.6% for the four environments with sand substrate). No correction of per cent mortality is possible, however, since it is not known whether the *Nereis* were distributed randomly with and without shells. I believe that there was a greater proportion of *Nereis* with megalopae having a shell.

In Experiments 2 and 3, per cent mortality was slightly greater for megalopae without shells (27.9 and 26.5%, respectively) than for those with shells (17.8 and 19.1%, respectively). In each case no significant difference in the numbers alive and dead under each treatment was detected when tested by an R × C contingency test. Although these two experiments are not directly comparable because of different sources of larvae and temperature-salinity regime, the overall mortality for both was 22.8%. This is markedly higher than in Experiment 1 and the salinity tolerance experiment described elsewhere (Roberts, 1971). This is attributed to the fact that larvae in Experiments 2 and 3 were not culled for the most active and healthy megalopae whereas in the other experiments only active megalopae were used.

Shell entry

The behavioral patterns of larvae entering a shell have already been described by Reese (1962) for laboratory reared megalopae of *P. longicarpus*, and by Hazlett and Provenzano (1965) for several other species, reared or collected from the plankton. Nothing can be added to their observations in this regard.

Shell entry can occur at any time during the megalopal instar. Of the megalopae which successfully entered shells, 46 to 50% did so within 24 hours and 82 to 93% within 48 hours. In each experiment, some larvae failed to enter the shell provided during the megalopal instar; 18.8% in Experiment 1, 21.6% in Experiment 2, but only 1% in Experiment 3. The disparity in this respect cannot be explained. These larvae did enter a shell immediately after molting to the first juvenile instar. Individuals deprived of a shell during the megalopal instar were given shells immediately after ecdysis and required only 5 minutes or less to gain entry.

Intermolt duration

Intermolt duration for megalopae with and without shells was compared for each experiment. For Experiment 1, no significant difference was found in the response of larvae to the various treatments in a preliminary R × C contingency test. The data were then pooled according to presence and absence of shell. Mean intermolt durations were 3.6 and 3.8 days for megalopae with and without shells, respectively. No correction was made for those larvae that failed to enter a shell during the test period.

In Experiment 2, correction was made for crabs that failed to enter a shell during the megalopal instar, these being added to the group not presented shells. The mean intermolt durations were 4.3 and 4.4 days for megalopae with and without shells, respectively.

Again in Experiment 3, there was no significant difference in intermolt duration for megalopae with and without shells. Mean intermolt duration was 4.9 days for megalopae with shells, 5.0 days for megalopae without shells. In this experiment, with only 1% of the larvae failing to enter a shell, no correction was made for these larvae.

DISCUSSION

Thompson (1903), testing the effect of various shells on megalopae of *Pagurus annulipes,* reported high mortality, ranging from 30 to 60% (excluding a group of 10 larvae in Series B_1). Megalopae without shells experienced 50% mortality (Thompson's stated value of 81% on page 186 seems in error), while megalopae with shells experienced only 44% mortality. In some experiments with a 3 or 4 day delay in presentation of the shell, the per cent mortality was even lower, rather than between 44 and 59% as would be expected. Nevertheless, Thompson concluded that megalopae without shells have a higher mortality than those with shells. Bookhout (1964) reached the same conclusion in his experiments with *Pagurus bernhardus.* In his experiments, larvae without shells experienced about 64% mortality (7 of 11) while mortality for larvae with shells was only about 7% (1 of 14).

In my experiments, mortality was unaffected by shell presence or absence. The discrepancy between the results of Thompson (1903), Bookhout (1964) and myself arises from the fact that in the experiments of the first two investigators megalopae were not isolated, whereas in my experiments they were. It has frequently been noted that megalopae in mass cultures inflict serious, often fatal wounds on one another. The difference in mortality for megalopae with and without shells in the experiments of Thompson (1903) and Bookhout (1964) reflects the role of shells in protecting the megalopae and later instars from predation, whether by members of their own species, or by other animals (as for example in Experiment 1).

The megalopa is initially an active swimmer, using its setose pleopods to propel itself, anterior end foremost, with the thoracic appendages held close to the carapace and with dactyli pointed forward much as in the brachyurans (Atkins, 1954). Swimming activity, though not quantified, definitely declined with time as was previously noted for *Birgus latro* (Reese, 1968). If a megalopa encounters a shell, it enters and swimming behavior ceases immediately if the shell is suitable. If no suitable shell is located, active swimming is nevertheless very infrequent after two days. By this time, the majority of megalopae are already housed in shells. The reduction in swimming activity is correlated with reduction of the pleopod musculature preparatory for metamorphosis as described by Thompson (1903).

Bouvier (1905) observed a very wide size range for what he believed to be conspecific hermit crab megalopae from the plankton. He postulated that failure to reach the bottom caused megalopae to delay metamorphosis and instead pass through a number of megalopal instars of increasing size. His identification of the megalopae as conspecific was assuredly incorrect since the megalopae ranged from 4 to 20 mm and differed in anatomical detail. His point is well taken that failure to reach the bottom or locate a suitable substrate with shell could result in delayed metamorphosis, as has been shown in a number of other organisms (see citations in the introductory remarks); however evidence for this phenomenon is lacking for hermit crabs.

Thompson (1903) examined the effect of dextral, sinistral, and straight shells, delayed introduction of shells, and shell absence on the intermolt duration of megalopae of *P. annulipes*. Intermolt durations in his experiments ranged from 4.4 to 5.4 days. He concluded that shell absence caused a longer mean intermolt duration (5.4 days) than shell presence (4.9 days, his Series A plus controls in Series B). However, a statistical comparison of his data reveals no significant difference in intermolt duration, which agrees with the results of the present experiments. The greater variability of Thompson's results may be a function of variable temperature and, in some cases, small sample size.

Reese (1968) reported that an unhoused *Birgus latro* megalopa failed to metamorphose after some 30 days whereas housed larvae metamorphosed after 21 to 28 days. Provenzano (1962) reported a *Coenobita clypeatus* megalopa that failed to metamorphose after 31 days in water although provided with a shell. This is the instar during which these species first emigrate to land. Clearly, megalopae of the terrestrial hermit crabs belonging to the family Coenobitidae have a long intermolt duration (20 to 30 days), but it is not clear from the results of Reese (1968) and Provenzano (1962) whether metamorphosis was actually delayed, and if so, whether the delay resulted from the effect of shell absence or immersion. Further experiments are needed to elucidate this and other points concerning the ecology of the megalopa of terrestrial species.

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Willard A. Van Engel critically reviewed the manuscript. Special thanks go to my wife, Beverly Ann, for her help in culturing many animals used in these experiments.

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SUMMARY

1. Mortality of isolated megalopae is unaffected by shell presence or absence. In mass culture, shell-less megalopae have a higher mortality because of cannibalism.

2. After 24 hours, 50% of the megalopae have entered a shell if available; after 48 hours, up to 93%. A few megalopae which failed to enter a shell did so immediately after the molt to the juvenile instar.

3. Intermolt duration was not significantly affected by shell presence or absence.

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THE DISTRIBUTION OF PHOSPHOARGININE AND PHOSPHOCREATINE IN MARINE INVERTEBRATES[1]

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Despite the wide morphological, ecological and functional diversity of the two or more million species of animals which exist on earth, comparative biochemical studies emphasize the basic similarity, at the molecular level, of all animal life. This is true both as regards chemical replicating mechanisms, on the one hand, and intracellular enzyme and co-enzyme systems which are particularly concerned with energy transfer, storage, and utilization, on the other.

The phylogenetic significance of the high energy phosphagens, phosphocreatine and phosphoarginine, was first suggested by the studies of Kutscher and Ackermann (1926) who proposed the principle that creatine was the phosphagen of vertebrate muscle and that invertebrates should therefore be termed "acreatinate." More recent studies by Yudkin (1954) and by Roche, Thoai and Robin (1957), who identified creatine phosphate as present in three other invertebrate phyla, have cast doubt on the original concept of Kutscher and Ackermann, and the inferences of Needham, Baldwin and Yudkin (1932) and Baldwin and Needham (1937), as to the phylogenetic implications of differential distribution of the two phosphagens. A recent review of Ennor and Morrison (1958) covered the scattered, later reports on the distribution of phosphocreatine in marine organisms. They concluded (page 665) that "phosphocreatine and phosphoarginine cannot be regarded as characteristic of the vertebrates and invertebrates, respectively, for, while phosphocreatine *is* a phosphagen characteristic of the vertebrates, no one phosphagen is characteristic of the invertebrates."

However, even the early work of Needham (1932) *et al.*, showed that creatine did occur in some of the echinoderms as well as the hemichordates, with the inference on their part, that this was evidence for a common ancestry for the echinoderms and the vertebrates. Other biochemical evidence by Bergmann, McLean and Lester (1943) and Bergmann (1949) and more limited embryological evidence by Breneman (1966) have also linked the echinoderms phylogenetically to the primitive chordates, particularly the hemichordates. However, the techniques of separation and identification of these two phosphagens, which have been employed in these earlier studies and upon which phylogenetic inferences were based, are either questionable or differ from one another significantly.

The present study was therefore undertaken in an attempt to resolve the question of the possible phylogenetic interrelationship of the echinoderms and primitive chordates, suggested both by embryological and by Bergmann's quite different biochemical line of evidence. Accordingly, the distribution of the two phosphagens in representative species of four major classes of the Echinodermata and in one common hemichordate was examined by more sophisticated methods of extraction and separation, and their identification by much more sensitive, analytical procedures than had been employed by most earlier workers.

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Materials and Methods

Determinations were made on the longitudinal muscles of the North Atlantic common sea cucumber, *Thyone briareus,* and the larger South Atlantic sea cucumber, *Ludwigthuria floridana,* on the dorsal skin of the North Atlantic starfish, *Asteria forbesi,* and of the local Florida starfish, *Echinaster sentus,* the complete rays of the brittle star, *Ophioderma brevispina,* the lantern retractors of the sea urchin, *Arbacia punctata,* the unfertilized eggs of *A. punctata* and of the Southern sea urchin, *Lytechinus variegatus,* the unfertilized eggs of *A. forbesi,* and finally, total body homogenates of the hemichordate, *Saccoglossus kowalevskii.*

Tissues were excised fresh, rinsed in sea water, quickly dried on toweling, weighed, and frozen in liquid nitrogen-cooled Elvehjem-Potter mortar with a stainless steel pestle. The homogenate was decanted and the homogenizer cup rinsed in cold 0.4 N perchloric acid into a cold stainless steel centrifuge tube. This was then centrifuged at 5° C, at 5000 rpm (3200 *g*) for ten minutes. The supernatant liquid so obtained was neutralized with cold 5 *N* NaOH and then quantitatively diluted to a known volume (10 ml) with cold, doubly-distilled water.

Arbacia eggs were collected by low voltage electrical stimulation and starfish eggs by means of the shedding hormone of Chaet (1966), in each case into 4 ml of cold doubly distilled water. After ten minutes to permit complete cytolysis, 4 ml of cold 0.8 *N* perchloric acid was added to each 4 ml of egg suspension. This mixture was then neutralized with cold 5 *N* NaOH and centrifuged cold at 10,000 rpm (12,800 *g*) and the supernatant liquid decanted and made to a known final volume with cold, doubly-distilled water.

Inorganic phosphate was determined by the method of Lowry and Lopez (1946) for "before" and "after" acid hydrolysis samples.

Appropriate acid hydrolysis consisted of heating known aliquots of neutralized brei, re-acidified with 0.2 *N* NaCl (1:1). For phosphocreatine hydrolysis, this involved nine minutes' exposure at 65° C. For phosphoarginine hydrolysis, the acidified brei was exposed for one minute in a boiling water bath. In each case, the previously heated acidified brei was cooled rapidly, prior to their separation and identification.

The techniques used in isolating and identifying the two guanidines include differential elution column chromatographic ion-exchange method for their separation and their identification and estimation by critical modifications of the Sakaguchi (arginine) and diacetyl (creatine) reactions. In each experiment, furthermore, a check on the procedure was made by side-by-side isolation and identification of creatine and arginine from standard solutions run through columns and similarly identified in parallel with the experimental biological material.

Known volumes of the cooled "before" hydrolysis samples were passed through one set of (three) $2'' \times \frac{5}{8}''$ Amberlite® columns and "after" hydrolysis samples through a second series of (three) similar columns, according to the method of Anderson, Williams, Krise and Dowben (1957), as follows:

Samples are poured into the first column containing previously-washed, strong anion exchange resin, IRA-401, and eluted at a rate of 1 ml per minute. This column retains most impurities but elutes both arginine and creatine upon washing with doubly-distilled water.

Column 2 contains IRC-50, a weak cation exchange resin, which binds the arginine but permits creatine to be freed upon elution with doubly-distilled H_2O.

Column 3 contains IR-120, a strong cation exchange resin, which holds the creatine.

Arginine is then eluted from the second (IRC-50) column with 50 ml of 2 *N* sodium acetate (pH 11.5) and the creatine similarly eluted with an identical volume of the same sodium acetate solution from the third (IR-120) column. In each case the eluted guanidine is collected into a 50 ml flask, at a flow rate of one ml per minute.

Arginine analysis

Arginine is determined quantitatively by the Sakaguchi method, as modified by Rosenberg, Ennor and Morrison (1956). A mixture of 2 ml of sulfosalicylic acid-oxime, 2 ml of the eluted sample and 1 ml of 2.5% NaOH is set aside in ice for 15 minutes and then warmed for 45 sec in room temperature tap water. One ml of hypobromite solution is then added and the optical density of the resulting solution is read immediately at 500 mμ in the B&L Spectronic 20.

Creatine analysis

This is the alkaline diacetyl-naphthol method of Barritt as adapted by Anderson *et al.* (1957), and Dubnoff (1957). The test mixture includes 1 ml of stock alkali, 1 ml of alpha-naphthol solution, 4 ml of the sample—previously adjusted to a pH of 9–10, with 5 *N* NaOH (approximately 0.4 ml of 5 *N* NaOH/25 ml sample) (or 4 ml of the standard or of doubly-distilled water) plus 1 ml of (Sigma) para-hydroxymercuribenzoate (3.6 g/200 ml). To this mixture is added 1 ml of diacetyl solution (1 ml of 1% stock solution which has been diluted 1–20 times in doubly-distilled water) and the optical density is read exactly 20 minutes afterwards at 525 mμ in the B&L Spectronic 20.

In each case replicates were determined for three "before" hydrolysis and three "after" hydrolysis samples and for three arginine and three creatine standards (0.25 mg/ml, diluted 1:50), all read against corresponding, doubly-distilled water blanks.

RESULTS

All data shown in Table I represent determinations made from specimens obtained fresh from the sea, *i.e.*, not stored over any period of time prior to isolation and determination of the phosphagens involved. Figures in parentheses indicate the number of positive tests for the guanidine or its phosphagen, out of the total number of experiments performed.

Results obtained clearly show that moderate levels of creatine phosphate occur in nine species of echinoderms, representing four of the five major classes of that

Table I

Distribution of arginine and creatine and their phosphagens in marine invertebrates

Specimens	Arginine	Arginine-PO_4	Creatine	Creatine-PO_4
Thyone briareus longitudinal muscles	± (8/11)	++++ (9/11)	± (8/11)	++++ (11/11)
Ludwigthuria floridana longitudinal muscles	++ (34/45)	++ (33/45)	+ (22/45)	+++ (31/45)
Asterias forbesi rays	± (10/18)	+ (13/18)	± (9/18)	++ (13/18)
ova	– (0/7)	– (0/7)	± (1/7)	– (0/7)
Asterias vulgaris rays	± (6/6)	+ (3/6)	± (1/6)	++ (5/6)
Echinaster sentus rays	+ (20/49)	+ (18/49)	+ (23/49)	++ (24/49)
Echinaster spinulosa rays	+ (2/5)	+ (3/5)	+ (3/5)	+++ (4/5)
Ophioderma brevispina rays	++ (1/6)	+ (1/6)	+++ (6/6)	+++ (5/6)
Arbacia punctata lantern protractors	– (0/13)	+ (1/13)	+++ (8/13)	+++ (12/13)
ova	– (0/8)	± (3/8)	– (0/8)	± (5/8)
Lytechinus variegatus ova	+ (5/7)	++ (2/7)	+ (3/7)	++ (5/7)
Saccoglossus kowalevskii	± (5/7)	++ (3/7)	± (2/7)	+ (1/7)

N.B. ± = 0 – 0.09 μeq/gram
+ = 0.10 – 0.49 μeq/gram
++ = 0.50 – 0.99 μeq/gram
+++ = 1.0 – 1.9 μeq/gram
++++ = 2.0 – μeq/gram

phylum. What is more significant is that these data were obtained from muscles in three studies and from tissues which are heavily muscularized, but from which excision of muscles *per se* is a technically difficult task, in the case of the remaining six species studied. Free creatine, on the other hand, was demonstrable in only small to trace quantities in all but two species, *viz.*, the rays of the brittle star, *Ophioderma*, and the purple sea urchin, *Arbacia*, lantern protractors showing relatively high amounts of that free guanidine.

The so-called "phosphagen of the invertebrates," arginine phosphate, occurred in moderate to high amounts only in the muscles of the two sea cucumbers studied, *Thyone* and *Ludwigthuria*. Moreover, this phosphagen and its free guanidine, was virtually absent from the lantern protractors or muscles of the echinoid, *Arbacia*, while being present in the unfertilized ova of both this sea urchin and the Southern form, *Lytechinus*. As for the hemichordate, *Saccoglossus*, only light to moderate amounts of *both* phosphagens were found in fewer than half of the experiments performed.

Discussion

These data are somewhat at variance from a number of earlier reports, summarized in the review by Ennor and Morrison (1958).

In this connection, further experiments might well be undertaken to confirm the identity of the phosphagen involved by the presence or absence of the appropriate (creatine or arginine) phosphokinase. However, as Ennor and Morrison (1958) point out, such experiments would assume a substrate specificity for each phosphokinase and for each species studied. Furthermore, such a series of experiments would require rather extended enzymatic studies of the optimal conditions for estimating enzyme activity for each species involved, as well as their substrate specificity characteristics. Collection of such data at this time would inordinately delay the publication of our own data, obtained by reliable and competent methods over a period of several years.

For the asteroids, we found, unlike Needham *et al.* (1932) (who reported only phosphoarginine in the starfish, *Marthasterias glacialis*, *moderate* amounts of both phosphocreatine and phosphoarginine) in the closely related species, *Asterias vulgaris*. Arnold and Luck (1933) also reported only *arginine* in several species of starfish, but by a method which suggested that this guanidine must be occurring as the phosphagen, rather than by direct determination of phosphocreatine.

As for the holothuroids, Kutscher and Ackermann (1926) reported only arginine in *Holothuria* and *Leptosynapta* and Meyerhoff (1928) only phosphoarginine in *Stichopus*. Baldwin and Needham (1937) inferred the presence of phosphoarginine in *Holothuria* sp., from their ability to synthesize the phosphagen from a solution of arginine, inorganic phosphate and muscle extract. Conversely, they assumed the *absence* of phosphocreatine from the *inability* of such extracts to synthesize the phosphagen from creatine and inorganic phosphate. Likewise, by indirect methods quite different from ours, both Baldwin and Yudkin (1950) and, more recently, Stephens, Van Pilsum and Taylor (1965) reported the absence of phosphocreatine in (what appears to be total body homogenates of) *Thyone briareus*. On the other hand, Verzhbinskaya, Borsuk and Kreps (1935), did report the presence of both phosphagens in the holothuroid, *Cucumaria frondosa*.

The virtual absence of both arginine and phosphoarginine in both echinoids studied, with rather appreciable amounts of creatine and phosphocreatine in *Arbacia punctata*, are in strong contrast to data of earlier reports. Thus, both phosphocreatine and phosphoarginine were reported by Arnold and Luck (1933) in *Strongylocentrotus franciscanus*, by Needham *et al.* (1932) in *Strongylocentrotus lividus* jaw muscles and by Baldwin and Yudkin (1950) in *Echinus esculentus* jaw muscles. However, all of the data by Baldwin and Yudkin (1950) were based on methods described by the authors themselves (page 617), as follows:

"although neither of these reactions is very specific, useful indications were nevertheless obtained"(?). Griffiths, Morrison and Ennor (1957) also reported both phosphagens in one echinoid, *Heliocidaris erythrogramma,* but only phosphoarginine in *Centrostephanus rodgersii,* which further indicates the relative variability of distribution of the two phosphagens, even within a single class of echinoderms.

In the brittle star, *Ophioderma brevispina*, only light to moderate amounts of arginine and arginine phosphate were found in only one of the six specimens studied, which compared favorably with the report of Baldwin and Yudkin (1950), who could detect only creatine phosphate in the arms and discs of *O. brevipina* and *O. longicauda*.

But for the primitive chordate, our own seven studies of *Saccoglossus kowalevskii* showed only traces of arginine, the presence of arginine phosphate in moderate amounts in at least three cases, and only traces to very light amounts of creatine and creatine phosphate in not more than two cases studied. This again is in contrast to the data of Baldwin and Yudkin (1950) in which only creatine phosphate could be reported by their methods, for the same species. Needham *et al.* (1932), on the other hand, reported both creatine and arginine phosphate, in the related hemichordate, *Balanoglossus salmoneus,* only creatine phosphate in the solitary urochordate, *Styela carnea*, but *neither phosphagen* in two other urochordates, the solitary form, *Ciona intestinalis*, and the colonial *Amaroucium constellatum*. Morrison, Griffiths and Ennor (1956) similarly reported the unequivocal absence of both phosphoarginine and the enzyme arginine phosphokinase, as well as arginine itself; on the other hand, they conclusively demonstrated the presence of creatine, phosphocreatine and creatine phosphokinase, in two species of tunicates.

All of these data for the echinoderms as well as the hemichordates are confounded even further by relatively recent findings that creatine phosphate is present in some sponges (Roche and Robin, 1954) and in at least two other invertebrate phyla (Roche *et al.*, 1957). Related comparative biochemical studies by Thoai (1957), by Thoai and Robin (1954), and by Thoai, Roche, Robin and Thiem (1953) supported by a recent report by Hobson and Rees (1955) who identified three new phosphagens of guanidyl derivatives (taurocyamine, glycocyamine and lombricine) in several species of annelids and other invertebrates which the authors suggest serve the same function as creatine in vertebrates.

In passing, one must comment on the use of the hydrolysis reaction alone as the criterion for inferring the presence of either phosphagen, without specific identification of the guanidine as such by some authoritative (hopefully specific) reaction. In this connection, Thoai *et al.* (1953), identified arginine phosphate and creatine phosphate by the usual, classical hydrolysis reactions, but they could not demonstrate the actual presence of released arginine for two species studied by a positive Sakaguchi reaction, nor released creatine, in the one case of *Nereis diversicolor,* by a positive Walpole test.

Finally, the possible implication of the biogenetic principle was tested by studies of the distribution of the two phosphagens in the (unfertilized) ova of the asteroid, *Asterias forbesi,* and the echinoid, *Arbacia punctata,* in relation to their distribution in the adult stages.

At best, the data obtained are equivocal, with creatine and arginine phosphate demonstrable in trace amounts in the ova of *Arbacia punctata,* but in moderate amounts in *Lytechinus* ova. Mende and Chambers (1953) and Chambers and Mende (1953), on the other hand, using the criterion of differential hydrolysis alone, without further separation of the two guanidines, reported the absence of phosphocreatine, but the presence of phosphoarginine by the Sakaguchi test following hydrolysis, in the unfertilized eggs of both *Asterias forbesi* and *Strongylocentrotus dröbachiensis.* The greatest significance of the data obtained in the present study include, first, the presence of creatine in all but two species studied and phosphocreatine in nine species of echinoderms (as well as in the single protochordate species). Secondly, in two species of echinoids, phosphoarginine appears to be replaced by what was formerly considered the phosphagen of the vertebrates, namely phosphocreatine, which occurs in moderately high concentrations in the lantern protractor muscles especially, where its role in energizing of muscle contraction must be logically assumed.

Thus, despite other evidence, some scattered embryological, some biochemical other than that involving the phosphagens (Hyman, 1955, 1959), the data obtained in this present study can only be interpreted as failing to confirm: (1) the hypothesis that invertebrates be considered acreatine, and (2) the existence of a phylogenetic relationship between the primitive chordates and aberrant echinoderms on the basis of biochemical evidence derived from the differential distribution of the two major phosphagens, phosphocreatine and phosphoarginine.

Indeed, the presence of creatine and phosphocreatine in a number of invertebrates, including annelids, as well as the existence of other more or less specific guanidine-derived phosphagens in other phyla like the Annelida, suggests that the distribution of phosphagens, in contemporary species must be only the results of parallel biochemical evolution.

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SUMMARY

1. The arginine, creatine, and related phosphagen content was determined in muscle-containing tissues of nine species of echinoderms, from four subphyla, and in one species of hemichordate. Similar determinations were made on the unfertilized ova of the starfish, *Asterias forbesi,* and of the two urchins, *Arbacia punctata* and *Lytechinus variegatus.*

2. Both phosphoarginine and phosphocreatine occur in moderate to large amounts in the longitudinal muscles of *Thyone briareus* and of *Ludwigthuria floridana.* Both phosphagens occur in light to moderate amounts in the rays of the starfish, *Asterias forbesi, Asterias vulgaris* and *Echinaster sentus,* with light amounts of phosphoarginine and large amounts of creatine phosphate in the rays of the starfish, *Echinaster spinulosa,* and of the brittle star, *Ophioderma brevispina.*

3. Light amounts of phosphoarginine and large amounts of phosphocreatine were also found in the lantern protractors of *Arbacia punctata.*

4. Light amounts of both phosphagens were found in total body homogenates of the hemichordate, *Saccoglossus kowalevskii.*

5. While both phosphoarginine and phosphocreatine are lacking in the unfertilized ova of *A. forbesi,* light amounts of both phosphagens are present in the ova of *L. variegatus* and trace amounts of both in the ova of *A. punctata.*

6. The data obtained fail to confirm (1) the early concept that invertebrates are "acreatinate" and (2) that differential distribution of the phosphagens, as such, can be employed as a criterion for confirming the possible phylogenetic relationship between the echinoderms and primitive chordates.

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SWIMBLADDER DEVELOPMENT AND FUNCTION IN THE HADDOCK, *MELANOGRAMMUS AEGLEFINUS* L.[1]

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Some of the earlier embryological studies on the teleostean swimbladder attempted to elucidate its evolutionary relationship to the lungs (*e.g.*, Spengel, 1904; Moser; 1904; Makuschok, 1913; Ballantyne, 1927), while others were done in recognition of its uniquely piscine nature (*e.g.*, Vogt, 1842; Ryder, 1884; Tracy, 1911; Maier and Scheuring, 1923). More recent investigations of swimbladder development have included studies of its initial inflation (*e.g.*, von Ledebur, 1928; von Ledebur and Wunder, 1937; Powers, 1932; Jacobs, 1938; McEwen, 1940; Wickler, 1959). However, these studies concentrated on morphological changes and concurrent larval behavior rather than on larval gas gland function.

The functioning of the swimbladder in adult fish presents some unique physiological aspects (Copeland, 1952; Fänge, 1953; Scholander, van Dam and Enns, 1956; Scholander, 1954; Copeland, 1969; Deck, 1970), which have not been extrapolated backward to early stages of development. Therefore, physiological observations are included where appropriate in this paper. Haddock was the species of choice since it has been thoroughly investigated as an important food fish. Much is known about its life cycle, vertical distribution of eggs and larvae, and rearing of larvae in the laboratory.

The swimbladder may originate in one of three ways. Most commonly it develops as a dorsal or lateral diverticulum of the gut, as in *Lepomis macrochirus macrochirus* (Duwe, 1952), but in *Coregonus palaea* (Vogt, 1842) it originates from the esophagus as a solid cell mass in which a cavity later appears. This cavity then grows down towards and establishes communication with the esophagus. Finally, it may originate as a solid cell mass, later to be invaded by an evagination of the gut, as in *Salmo salar* (Hoar, 1937). Sinnce Meek (1924) found the swimbladder of *Gadus morhua* (=*callarias*) to originate as a diverticulum of the gut, a major objective of this study was to determine its mode of origin and course of development in another member of the Gadidae, the haddock.

At least two methods exist by which the swimbladder may be first inflated. Many larval physoclists swim to the surface and gulp air at or shortly after hatching, while they are still morphologically physostomous. This air may somehow stimulate production of gas by the gas gland (Jacobs, 1938), which in some species is already present in connection with the rete mirabile. On the other hand, the duct may degenerate prior to hatching or, if present, its lumen may be closed. Fishes in this category would not swallow surface air, and some internal mechanism would have to stimulate gas production. Thus an attempt was made to determine the method of swimbladder inflation in the haddock.

Swimbladder gas gland cells in the adults of a number of species store glycogen when not secreting and metabolize it during periods of activity (Copeland, 1952;

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[1] This work forms part of a dissertation submitted in partial fulfillment of the requirements for the Ph.D. degree at Boston University, Boston, Massachusetts 02215.

Fänge, 1953). No work of this kind has been done on larval fishes. I thought it possible that, prior to initial inflation, the gas gland might concentrate glycogen which could be used as the energy source for the process. A third objective therefore was to test for the presence of glycogen in gas glands of larval haddock.

Adult and juvenile haddock live near the ocean floor. The eggs are spawned at the bottom between February and May but develop in the upper layers of the ocean. The larvae remain pelagic until August or September, when they return to the bottom (Miller, Colton and Marak, 1963). An attempt was made to determine what role, if any, the swimbladder plays in the vertical distribution of the larvae during their pelagic phase.

Methods and Materials

About 250 fertilized haddock eggs were incubated at 5.5° C in quart jars three-fourths filled with seawater, 50–75 eggs to a jar. Aeration was facilitated by twice-daily stirring. Temperatures were checked daily.

Every morning, from the time of blastopore closure (day 7) on, 10–15 eggs were fixed for 24 hours at 27° C in 10% Lillie's neutral buffered formalin. After removal of the vitelline membranes, specimens were dehydrated and cleared in graded alcohols, toluene, and cedarwood oil, and bathed and embedded in Paraplast. Transverse serial sections at 4 μ were stained routinely with Harris' hematoxylin and eosin.

The behavior of the larvae upon hatching was observed and recorded. A five-gallon polyethylene tank was filled with two gallons of seawater, and the whole cooled by circulating tap water. The temperature stabilized at 10° C and the larvae underwent a 1.3° C per day rise in temperature until 10° C was reached on the fourth day of larval life, when they were introduced into the tank. The culture temperatures gradually rose from the initial 10° to 13° C because of seasonal warming of the tap water.

From the day after hatching on, the larvae were given one teaspoon daily of *Artemia salina* nauplii. Due to the large surface area of the tank, twice-daily stirring provided the only further aeration.

A few larvae died each day. This plus periodic sampling depleted their numbers so that fixations were not done beyond larval day 12. Visual observations were continued on the remaining larvae until day 19.

The 10 larvae in each sample were fixed, dehydrated, cleared, bathed and embedded using the same procedure as for the eggs. Four larvae per sample were sectioned transversely at 4 μ and stained with Harris' hematoxylin and eosin. The other six were sectioned transversely at 5 μ and treated using the PAS technique with a hematoxylin counterstain; three of these six were incubated with malt diastase prior to staining. The diastase treatment (and subsequent PAS on diastase-treated slides) was performed by Histology Service Inc., Philadelphia, Pennsylvania.

Out of 60 plankton sampling attempts I made while on board the R/V ALBATROSS IV (cruise 68–8), five haddock larvae were obtained. These were examined for swimbladder glycogen using the method described above.

A number of other larvae, collected during surveys made on the R/V Albatross IV in 1967 and 1968, were obtained as preserved material from the Bureau of Commercial Fisheries, Woods Hole, Massachusetts.

Results

Embryogenesis took 17 days at 5.5° C. Four of the 250 eggs died during this period. Larval mortality rose sharply after yolk sac absorption, although the larvae were feeding on *Artemia* nauplii. The last larva died 19 days after hatching. (Previous attempts to rear haddock larvae in the laboratory had been unsuccessful in that the larvae did not survive beyond 21 days; David Miller, Bureau of Commercial Fisheries, Woods Hole, Massachusetts, personal communication).

Morphology of laboratory-reared eggs

Day 11. The swimbladder anlage appeared on day 11, when embryogenesis was two-thirds completed. It showed as a shallow evagination of the dorsal gut wall at the level of the pectoral fins. A narrow lumen opened into the gut opposite and just posterior to the connection of the gut with the liver.

The gut wall in this area was composed of stratified columnar cells surrounded by one to two layers of mesenchyme. These were enclosed in turn by a thin layer of fibroblasts. The bladder anlage was constructed similarly, but there was only one layer of columnar epithelium lining its lumen. There was little indication of blood vessels serving the swimbladder region.

Day 12. The anlage had become a narrow inverted U with a distinct lumen patent throughout its length. No distinction could be made between pneumatic duct and swimbladder proper. The inner lining of the anlage consisted of a single layer of tall columnar cells; near its origin from the gut, however, another columnar layer appeared, reflecting the stratification of the cells of the gut lining. The cytoplasm of the columnar cells was intensely basophilic, a characteristic which persisted throughout embryogenesis and during early larval life.

Around the columnar lining there was a pronounced agglomeration of irregularly-layered, undifferentiated mesenchymal cells. Venous sinusoids and capillaries were occasionally observed in the mesenchymal mass.

Day 13. The inverted U of the swimbladder had elongated somewhat more. The mesenchymal cells had proliferated in an antero-posterior direction, although they were still evenly distributed up and down the outgrowth. The layers of fibroblasts and fibers surrounding the swimbladder had increased in number and in thickness. Venous sinusoids and capillaries appeared with increasing frequency, especially in the outer layers of the swimbladder and at its base on the side nearest the liver.

Day 14. A distinction was apparent between pneumatic duct and swimbladder proper, due in part to the lumen's enlargement at its distal end. Also contributing was a new concentration of mesenchyme around the distal end of the outgrowth, forming a ball surrounded by several layers of fibroblasts and collagenous fibers and enclosed by the layer of pigmented peritoneum characteristic of the adult condition.

The swimbladder had become more dorsal in position. The mesenchyme had increased in bulk antero-posteriorly and had begun organizing into layers.

Day 15. In contrast to the duct lumen, the swimbladder cavity had continued to expand. The concentration of fibroblasts and fibers had increased over the anterior and posterior ends of the bladder. Within the mass of mesenchyme, more capillaries and one venule were present, situated as before on the side of the swimbladder near the liver.

Day 16. The swimbladder cavity had undergone pronounced expansion in a dorso-ventral direction. The pneumatic duct was now seen to emerge from the right side of the bladder. Its lumen was patent throughout and its walls were intact.

A well-developed basement membrane appeared beneath the epithelial cells lining the cavities of swimbladder and pneumatic duct. Several venules were present in the mesentery between swimbladder and liver.

Day 17. The swimbladder had lengthened antero-posteriorly, so that the pneumatic duct entered it approximately midway along its length.

The structure of the duct had not altered significantly since its first appearance. A single row of cuboidal cells bordered on the patent lumen. These were enclosed by a layer of small cuboidal mesenchymal cells which were continuous around the swimbladder. The mesenchyme was surrounded by one or two layers of loose fibrous connective tissue and a layer of pigmented peritoneum.

Morphology of laboratory-reared larvae

Day 1 (average total length = 3.5 mm). By the time of hatching, the swimbladder had grown more anteriorly. The pneumatic duct thus appeared far posterior in position.

Day 3 (average total length = 3.8 mm). Swimbladder growth in an anterior direction had continued, and the tissue layers showed signs of increasing organization. At least two layers of columnar cells now lined the cavity, and the mesenchyme was now compacted into distinct layers that closely surrounded the epithelial lining.

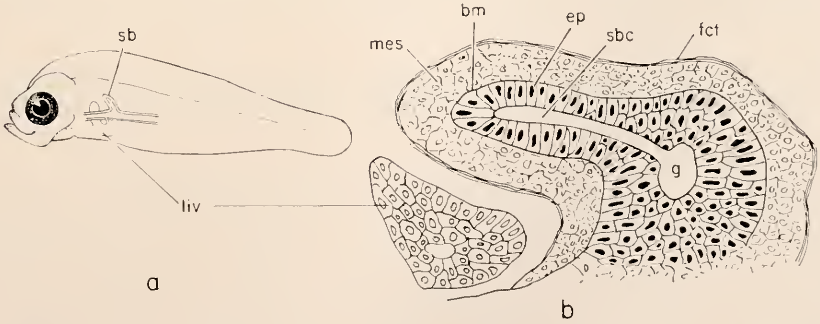

FIGURE 1. (a) Diagram of swimbladder position, newly hatched *Melanogrammus aeglefinus.* Dorsal is at top in all figures. (b) Cross-section through swimbladder region of a day 12 embryo. Abbreviations are: sb = swimbladder, liv = liver, mes = mesenchyme, bm = basement membrane, ep = epithelium, sbc = swimbladder cavity, fct = fibrous connective tissue, g = gut lumen; × 156.

The rounded bladder tapered caudally to meet the pneumatic duct. The duct appeared short, thick, and fairly straight, passing through the fibrous coats before turning to enter the posterior end of the swimbladder cavity on the right side. The duct lument was patent throughout and showed no sign of narrowing. However, the layer of mesenchymal cells distal to the cuboidal epithelium lining the duct appeared somewhat disorganized, and this proved to be the first sign of duct degeneration.

The cytoplasm of the epithelium and mesenchyme was now much more acidophilic; this increased in later stages.

Day 6 (average total length = 4.3 mm). The yolk sac had been absorbed by now and feeding had begun. The swimbladder had assumed a shape generally similar to that observed after inflation.

The cavity itself varied in shape among specimens examined, but no changes in size were evident. It was lined by stratified cuboidal epithelium, distal to which were polygonally-shaped mesenchymal cells and some blood vessels.

The epithelial cells bordering the duct lumen appeared flatter, and the structure of the mesenchymal layer more chaotic, although the lumen still exhibited a uniform diameter. No structure resembling a sphincter was evident in the duct region. Although the duct was located at the anteriormost end of the midgut, the common bile duct had appeared to enter the midgut anteriorly to it. This was now seen to have been caused by a craniad expansion of the midgut at this point.

This day marked the appearance of the rete mirabile, which resembled a collar almost completely surrounding the bladder and set on a diagonal to it. Posteriorly, the rete extended past the entrance point of the pneumatic duct to cover the bladder's posterior wall. Erythrocytes were present within its vessels, which at this stage were mainly venules and capillaries. The rete extended poorly-defined, branching projections into the main bladder mass. This arrangement suggested that seen in older larvae and adults, where the rete resembles a tree whose vascular branches are traced by singly-layered cuboidal glandular cells and whose trunk is composed of thick bundles of parallel blood vessels. The rete was surrounded by a layer of fibrous tissue which was continuous around the bladder.

Day 10 (average total length = 4.8 mm). A single layer of columnar cells now appeared to line the swimbladder cavity. This lining had become somewhat folded.

The mesenchyme distal to the lining had proliferated and now enclosed many blood vessels containing erythrocytes. More erythrocytes were present within the rete.

The cells lining the pneumatic duct were still more flattened and appeared to form a syncytium. The basement membrane had disappeared.

Day 12 (average total length = 4.8 mm). The folds of the cavity lining were more pronounced, suggesting the convoluted appearance of the mature gas gland. The cells were larger and less stratified, with markedly acidophilic cytoplasm. In some areas there was a proliferation of very small cells immediately beneath the prominent basement membrane. The rete was packed with erythrocytes.

The lumen of the pneumatic duct had altered in diameter. Although unchanged at the swimbladder end, it was extremely narrow where it opened into the gut. In six specimens it appeared open and in two others it appeared closed.

The mesenchymal cells had disappeared from the duct wall, leaving only a chaotic syncytial epithelium.

Morphology of collected specimens

Collected larvae were obtained from the Bureau of Commercial Fisheries and from seining by the author. Larvae in the first category measured 4–17.5 mm while in the second group four averaged 6.3 mm and one measured 8.0 mm in total length.

Major features of swimbladder development in these specimens were cavity expansion, increasing antero-ventral localization of most of the cuboidal (?glandular) epithelium, rete development, and the appearance of accessory tissue layers. Swimbladder development was arbitrarily divided into three stages on the basis of larval size; *viz.* 4–5 mm, 5–10 mm, and 10–17.5 mm.

In laboratory-reared larvae of 4–5 mm the cavity was small and the lining deeply folded. In collected specimens of similar length the cavity was noticeably larger and the folds not as deep. Cuboidal epithelium surrounded the cavity, as did rete vessels, except most postero-dorsally where a small area was lined by thin squamous epithelium. Occasionally vacuoles were evident within the cuboidal cells.

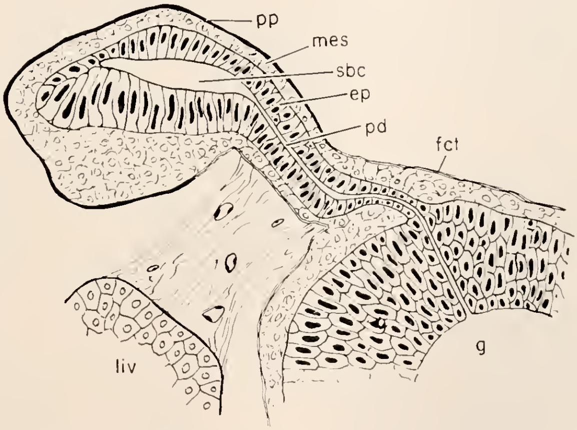

FIGURE 2. Cross-section through swimbladder region of a day 16–17 embryo. Abbreviations are: pd = pneumatic duct, pp = pigmented peritoneum; × 156.

In 5–10-mm collected larvae the cavity was still further expanded. The swimbladder appeared teardrop-shaped in lateral view, tapering posteriorly. The trunk of the rete ran along the outside of the ventral swimbladder wall and divided in two before entering the cavity anteriorly. Its vessels were most abundant here, but it also sent branches caudally along the lateral walls of the bladder. This organization apparently determined the localization of the cuboidal epithelium, which had proliferated over the anterior and antero-ventral walls of the bladder and in some specimens covered a small portion of the antero-dorsal wall as well. In these areas it was complexly folded around elements of the rete. From here it extended as two tongue-shaped masses running posteriorly along the lateral walls almost to the end of the cavity. In these places the epithelium was unfolded and each cell appeared to be in contact with a rete vessel. Intra- and intercellular vacuoles were present. Caudally there was a slight decrease in the size of the cuboidal cells. The tongue-shaped masses tapered slightly at their caudal ends. The dorsal wall was almost uniformly thin, exhibiting only one layer of squamous cells, but the ventral wall anteriorly sometimes showed a few layers of squamous epithelium underlying the cuboidal tissue. Further posteriorly, its lining cells were transitional between cuboidal and squamous, and its caudal end was covered by a very thin sheet of squamous epithelium.

The cavity in third-stage swimbladders was very large, accounting in part at least for the size reduction and sharp tapering of the lateral masses mentioned earlier. Third-stage bladders also exhibited two to four layers of circular smooth muscle ventrally. These seemed to diminish dorsally, and in fact the dorsal wall was uniformly thin and histologically almost featureless.

In the third stage, as in the second, the anterior concentration of cuboidal epithelium was at least partly divided into two masses, following the division of the rete where it entered the bladder.

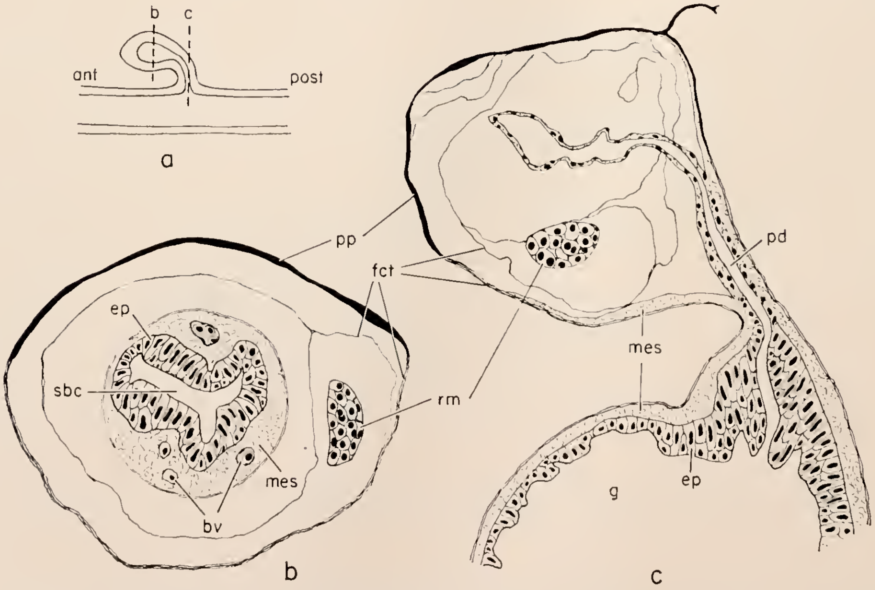

FIGURE 3. (a) Diagram showing area of sections (b) and (c). (b) cross-section through swimbladder cavity of a day 10 larva. Abbreviations are: bv = blood vessel, rm = rete mirabile. × 156. c, cross-section through region of pneumatic duct, day 10 larva; × 156.

The state of the pneumatic duct in laboratory-reared larvae averaging 4.8 mm has been described. In collected specimens, the duct was closed at the opening into the gut in three 4.5-mm individuals, and had become composed largely of connective tissue fibers. A small depression where the duct opened into the swimbladder was apparent in specimens up to about 7.0 mm in length, and was always to be found on the lower right bladder wall. In larger specimens only a few connective tissue strands were left to represent the duct. The lumen first closed at the opening into the gut, but a small thickening of the outer gut wall marked its former position even in a 17.5-mm specimen.

Glycogen in laboratory-reared larvae

All these animals had uninflated swimbladders. The concentration of glycogen in swimbladder epithelium increased from the time of hatching on, reaching a maximum on day 10. The mesenchymal cells did not exhibit glycogen until after yolk sac absorption was completed, after which they showed an increasing glycogen concentration. Other PAS-positive material was absent from the mesenchyme at hatching, most evident on day 6, and then declined.

All PAS-positive material in both epithelium and mesenchyme remained evenly distributed in the cytoplasm throughout the course of observed development.

Glycogen in collected larvae

These larvae had inflated swimbladders. Specimens obtained from the Bureau of Commercial Fisheries had been kept in formalin for long periods of time and so could not be examined for glycogen. The glycogen content in inflated swimbladders was thus determined only for larvae collected by the author. In swimbladder epithelium of these specimens, glycogen was absent from the paravascular areas but was abundant elsewhere in the cells.

Behavior

Hatching in the laboratory occurred throughout the water column, not just at the surface. None of the larvae swam towards the surface at this time. The animals were not seen to swallow air or to attempt to do so at any time in the course of their subsequent development.

Discussion

I. *Role of swimbladder during larval pelagic phase*

Colton (1965) demonstrated that while haddock eggs were present throughout the water column, they were most abundant at the surface, decreasing linearly with depth. Miller *et al.* (1963) showed that this was not the case with the larvae, although since spawning occurred earlier than usual in that year, only 5–10% of the total catch consisted of newly-hatched animals. The water column was sampled to 75 meters, but over 80% of the larvae from almost 4.0 mm to 21 mm in total length were found between 10 and 40 meters. They constituted two groups, the first containing larvae of 8.0 mm and less, and the second including those over 9.0 mm. Larvae in the first group were dispersed over a greater depth range than those in the second, exihibiting a random distribution with no region of maximum density. However, over 80% of the larger larvae were concentrated within the thermocline. No vertical migration was observed for any of the animals, although further studies were deemed necessary.

It is possible that the swimbladder becomes functional when the larvae reach about 9.0 mm, with the result that they may better adjust their density and thus stratify out at some preferred level, in this case the thermocline. I examined 40 collected larvae measuring 4–17.5 mm, half of which were 4–9 mm. In all specimens the swimbladder was at least partly inflated, and in larvae of 5.0 mm and over it occupied the same percentage of the body area in cross-section as it did in the adult. Therefore the swimbladder was probably functional in larvae less than 9.0 mm in length. A possible role for it is discussed below.

The thermocline in the area studied by Miller *et al.* (1963) generally does not form until April or May, and before this time most of the larvae average less than 9.0 mm in length (Robert Marak, Bureau of Commercial Fisheries, Woods Hole, Massachusetts, personal communication). Although admittedly little is known of the ability of the small larvae to cross barriers posed by temperature gradients and current velocities, their swimming ability is probably relatively undeveloped. Their random vertical distribution might thus indicate a passive dispersal caused by hydrographic factors that thoroughly mix the water column. However, the inflated swimbladder decreases the density of the fish, so that as the winter mixing decreases, the smaller larvae might be carried up into a range able to be affected by the forming thermocline. By the time of its formation, the larvae would be larger and of greater swimming ability, and would be able to maintain themselves among food concentrations such as exist within it.

II. *Time of swimbladder inflation*

The pelagic phase of the haddock includes animals from 3.5 mm (hatching size, Bigelow and Schroeder, 1953) to about 100 mm (Miller *et al.*, 1963). It is within the pelagic period that the swimbladder begins functioning as a hydrostatic organ.

Examination of the liver and gut, as well as body length measurements, revealed a disparity in development between laboratory-reared and collected larvae in favor of the latter. This may have been due to a lack of optimum food and temperature conditions in the laboratory. Laboratory animals were reared at temperatures averaging up to five degrees higher than those to which larvae less than 8.0 mm would naturally be exposed. Furthermore, most collected specimens had ingested several planktonic forms whereas laboratory-reared larvae were given only *Artemia* nauplii.

Swimbladder inflation commonly occurs either upon hatching as in *Hippocampus* (Jacobs, 1938) or just after yolk sac absorption as in some salmonoids (Tait, 1960). Yolk sac absorption was finished and swimbladder inflation completed or nearly so in all collected larvae. Although laboratory-reared specimens of equal size (4–4.5 mm) had completed yolk sac absorption, they never showed inflated swimbladders. Thus this study did not furnish a definite time for initial inflation, but such evidence as is available (hatching occurs throughout the water column, not just at the surface; laboratory-reared larvae do not swallow air upon hatching; the rete does not appear until after yolk sac absorption) indicates that it occurs soon after yolk sac absorption rather than upon hatching.

III. *Mechanism of swimbladder inflation*

If swimbladder inflation resulted from swallowing surface air, larvae less than 4.5 mm might be most abundant at the surface. However, Miller *et al.* (1963) found small larvae to be concentrated well below the surface. Although in that study the larvae were not observed at the time of hatching, hatching may occur anywhere in the upper 20 meters (Colton, 1965) and did not always occur at the surface in the laboratory. Laboratory-reared larvae were not observed to gulp air upon hatching or at any time thereafter. However, the natural behavior of the larvae upon hatching can still only be conjectured. Moreover, since collected larvae 4–4.5 mm all had closed pneumatic ducts, while in laboratory animals of equal size the duct remained open, the state of the duct gave no clue as to method of inflation. Nevertheless, use of a mechanism other than swallowing air is suggested.

An interesting alternative was suggested by Powers (1932) who stated that initial inflation should normally occur before gas gland function ensued. Although not certain how this would come about, he postulated the disintegration of certain organic materials within the bladder epithelium, producing carbon dioxide within its lumen. Some support for this was given by McEwen (1940) and Johnston (1953).

McEwen found that the swimbladder lining in *Hemichromis bimaculatus* was at first composed of cells resembling those lining the gut, but within 24 hours they became greatly vacuolated and expanded to fill the bladder lumen. Two to nine hours later the cells had transformed into a flat epithelium, leaving a gas-filled bladder lumen. McEwen thought that this gas came from the vacuoles, but as the bladder's cross-sectional area was not as great after lumen obliteration as after cellular flattening, he suggested that additional disintegration of cellular material was going on continuously during this time.

Johnston (1953) found similar vacuolated cells temporarily comprising the ventral swimbladder epithelium in *Micropterus*. Although *Micropterus* did not swallow air to fill its bladder, the organ increased in size and in gas volume, and the pneumatic duct remained open during inflation. Johnston thought it possible that some of the initial gas resulted from digestion.

How the presence of gas released from vacuoles eventually stimulates gas gland activity is unknown. The problem is further complicated in *Hemichromis* and *Micropterus*, neither of which possesses a rete at the time of initial inflation. Further studies in this area would be desirable.

Swimbladder inflation in the haddock was not observed to occur in this manner. Vacuoles in swimbladder epithelium only appeared after inflation. Moreover, no cellular expansion was seen.

It is possible that glycogenolysis is the major energy source for production of swimbladder gas in the haddock. Support for this statement derives from studies showing that much of the gas in the swimbladder of *Gadus morhua*, another gadid, could be derived from glycogenolysis (Fänge, 1953). Moreover, in some other genera (*Perca*, Fänge, 1953; *Fundulus*, Copeland, 1952), glycogen disappeared from gas gland cells during inflation and reappeared during gas resorption. Histochemical studies on these fishes revealed a distribution of glycogen in the gas gland similar to that observed here in the inflated haddock swimbladder.

As epithelial cells in the uninflated haddock swimbladder were found to concentrate glycogen, it is proposed that the initial gas supply is derived from glycogenolysis. The intra- and intercellular vacuoles observed in the inflated bladder's epithelium may have been associated with glandular function rather than with intracellular disintegration of organic materials as postulated for other species by Powers (1932). The rete was developed well before inflation, and made apparent contact with each epithelial cell examined. Additional data necessary to support this proposed mechanism could most profitably accrue from studies of gas gland ultrastructure.

While glandular function may provide the initial gas, how the gas gland cells are first stimulated to function remains unanswered. It is possible that secretion begins when the concentration of specific metabolites in the blood passing through the rete reaches a certain level, the buildup perhaps resulting from the onset of feeding.

It is of interest that mesenchymal cells just distal to the swimbladder lining concentrated glycogen. These cells differentiated into blood vessels of the rete. Glycogen may have been an energy source for cellular differentiation (Donald Patt, Department of Biology, Boston University, Boston, Massachusetts, personal communication), further illustrating the need for ultrastructural studies of differentiating tissue.

Oval development was not studied, but it is doubtful that the oval in the haddock develops from the degenerating pneumatic duct as in *Opsanus* (Tracy, 1911). Inflation resulted in a uniformly thin-walled roof even in 5.0-mm larvae, and an oval was not seen in any specimen. Furthermore, the opening of the duct into the bladder was always found on the lower right swimbladder wall. It seems unlikely that the entire swimbladder would shift position by ninety degrees in order that this opening be located dorsally. Some support for this view is given by Srivastava (1957), who found the duct coexisting with the oval in several species of Mugilidae.

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SUMMARY

The swimbladder in the haddock appears on the 11th day of embryogenesis as a dorsal outgrowth of the gut just posterior to the liver diverticulum. In later stages its true position just anterior to the liver becomes visible.

Swimbladder inflation occurs very soon after yolk sac absorption. At this time the larvae measure 4–4.5 mm and have begun feeding.

Haddock larvae probably do not swallow air to fill their swimbladders. The initial gas volume may be derived from glycogenolysis, as glycogen is present in substantial amounts in uninflated swimbladder epithelium. Glycogen is absent from the paravascular portions of the epithelium in inflated swimbladders but is well-represented elsewhere in the cells.

It is proposed that the inflated swimbladder in larvae less than 8.0 mm serves to decrease their density and thereby carry them up towards the level of the forming thermocline. By the time the thermocline is well-established, the larvae are larger and of greater swimming ability, and with the aid of a functioning swimbladder should be able to maintain themselves within it.

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AGE AND GROWTH OF *LOLIGO PEALEI*, A POPULATION STUDY OF THE COMMON ATLANTIC COAST SQUID[1]

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Loligo pealei (Lesueur, 1821) has been the subject of continuing scientific interest, especially at the institutions in Woods Hole, Massachusetts. The species has supported a small domestic bait fishery amounting to about 1000 metric tons per year over the period 1879–1967 (Lyles, 1968). The Russians have investigated the possibility of developing an offshore fishery (Vovk, 1969) and the Japanese began a large-scale harvest of the species in the New York Bight with 14–15 vessels during the winter of 1969–1970. Bureau of Commercial Fisheries market news release indicated that the Japanese fleet took approximately 13,000 metric tons of *L. pealei* during a three month season in the first year alone. This greatly increased exploitation and a developing concern for marine populations in general require a practical means for aging *L. pealei* to facilitate studies of its life history.

A. E. Verrill (1882) reported on the rate of growth and size of *L. pealei* to refute what he considered a commonly held opinion that all squid are annual animals. This was the first quantitative contribution of its kind in squid biology and it exerted considerable influence on subsequent teuthological studies. Verrill based his report on a logical assemblage of size data from preserved specimens collected at various localities along the New England coast during the warmer half of the year. Recognizing the difficulty of identifying age groups among these specimens, he tabulated generous size ranges which suggest a single, diffuse class each year and a longevity of three or four years. Difficulty in confirming Verrill's suggested growth rate for young *L. pealei* has been reported (Summers, 1968). In my experience, Verrill's growth scheme lacks sufficient precision to be useful in analyzing the size structure of this species and introduces problems which are inconsistent with present knowledge of squid biology.

In general, squid lack nautral age markings (annuli), do not survive well in capitivity (Summers and McMahon, 1970 and unpublished) and are poor prospects for ordinary tagging studies. As a result, reports on the growth of squid usually are based on their population dynamics. Commercial sources have frequently been employed in these studies with a probable introduction of sample bias (Tinbergen and Verwey, 1945; Mangold-Wirz, 1963; Fields, 1965 and others). Various statistics have been used to describe size classes of squid; in a probable series of increasing statistical reliability, these include: record size (Choe, 1966; Clarke, 1966), size range (Verrill, 1882; von Boletzky *et al.*, 1971), modal size (Tinbergen and Verwey, 1945; Rao, 1963; Haefner, 1964; Fields, 1965), mean size (Fridriksson, 1943; Squires, 1957 and 1967; Jaeckel, 1958; Mangold-Wirz,

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1963) and fitted size distributions (Murakami and Shindo, 1949; Summers, 1968). Size ranges are of little value without a knowledge of sample size, bias and distribution. Modal sizes must be interpreted and are subject to chance "noise" in the data. Mean sizes are inappropriate for mixed age groups and perhaps for mixed year classes. Finally, to be meaningful, fitted size distributions must reflect the biological events regulating natural size structure.

It was the purpose of this study to use population dynamics techniques for a new description of age and growth in *L. pealei*. Attempts to provide contrasts with reports in the literature are intentional in view of Verrill's precedent with this species. As far as possible, systematic sampling was employed. Gear of estimable sampling bias was used and the data were analyzed by a sensitive (non-prejudiced) statistical technique in an attempt to identify valid age groupings.

MATERIALS

Quantitative sampling of *L. pealei* extended from August 1967 to July 1970 and a qualitative survey continued into November of 1970. Except for newly hatched (planktonic) squid and materials used for comparative purposes, all samples resulted from daytime collections employing large otter trawl nets. Offshore winter collections made between Georges Bank and Cape Hatteras in March and April of 1967 and 1968 were reported previously (Summers, 1969). Fifty-two collections were made during the inshore season (May through November) near Woods Hole; most of them in Menemsha Bight in southern Vineyard Sound (a description of this station appears in Summers, 1968). Inshore collections were made in all three years with either a #35 otter trawl or a 45–65 Long Island Sound balloon trawl (Summers, 1968; Summers and McMahon, 1970). Plantonic squid were taken near the surface with one-half meter plankton nets (860 micrometer mesh size) on several occasions in July and August 1967–1969. Subsurface collections of planktonic squid were made with a six foot Isaacs-Kidd midwater trawl (fine mesh cod end) on July 17, 1969 and with paired, one-fifth meter "bongo" nets (505 micrometer mesh size) on July 2 and 14, 1970.

Squid collections from some large catches were subsampled volumetrically, *i.e.*, a large portion of the catch was spilled over a sample bucket which held a minimum of 50 adult squid. All measurements and dissections were made on fresh, unpreserved animals. Dorsal mantle length was recorded to the nearest whole centimeter, sex and sexual maturity were noted for every individual excepting young-of-the-year squid with dorsal mantle lengths less than about six centimeters. Males were considered mature if spermatophores were present in Needham's sac (spermatophoric sac) and females were classified mature if the ovary was expanded and loose eggs were found in the oviduct. These maturity indices are potentially more subjective for females than for males and are not considered a positive indication of breeding activity at the time of capture. During the breeding season, we recorded as mature a number of small squid (10 cm dorsal mantle length or less) which may not have participated in the breeding activity and probably could not have produced a full compliment of fetrilized eggs without further development. Records of 15,132 squid resulting from the above sources were utilized in this report.

Several miscellaneous collections were used for comparative purposes. These resulted from the operation of a fishtrap (Summers and McMahon, 1970), squid jigging under lights, stomach contents of predatory fish and trawl samples from surveys conducted by the Bureau of Commercial Fisheries (now, National Marine Fisheries Service).

METHODS

Data from each sample were tabulated in one of three categories: (1) males, (2) females and (3) young-of-the-year squid. Individuals in the last category were all sexually immature and generally were not sexed; occasional dissection, however, indicated a consistent 1:1 sex ratio. Further analysis was carried out when data from twenty or more squid were present in a particular category. The actual numbers of collections analyzed and average numbers of specimens per category, respectively, for the inshore sampling were as follows: males, 39 and 56.3; females, 34 and 58.6; young-of-the-year, 27 and 157.3. All categories were not sufficiently numerous to qualify for inclusion in each collection, so the inshore data represents 45 collecting dates and 8,437 squid distributed over three, 7 month periods.

Size data were subjected to a size class separation using the method described by Harding (1949) and extended by Cassie (1954). Polymodal frequency distributions were graphically fitted by normal distributions on probability paper, providing estimates of mean, standard deviation and relative abundance for each mode. Normal size distributions were assumed for sexed squid and lognormal distributions for young-of-the-year squid to accommodate a positive skew in their sizes (Summers, 1968). Mean and logmean mantle lengths were weighted by the relative abundance for analysis and standard deviations were compared for consistency of class separation. Weighted mean sizes were tabulated for each size class by category; these were pooled by cruise for the winter collections and by month for inshore sampling. Initial phases of a second size class separation were carried out on the pooled data to locate intersections between major size clusters at different times of the year. Winter data were not considered to be weighted comparably to inshore collections because of differences in fishing gear and depth stratification of *L. pealei* in the winter (Summers, 1969); their use was restricted to qualitative aspects in further analysis.

RESULTS

Results of the data treatment are summarized in Figure 1. Heavy vertical lines in the figure represent the monthly range of size class means and oblique lines bordering the stippled area indicate the intersections of principle size clusters (results of the first and second size class separations, respectively). Age groups are identified by size clusters under the assumption that breeding is seasonal. Age is inferred by following population growth backward in time to hatching. Quantitative data in Figure 1 are a composite for males and females assuming a 1:1 sex ratio (the approximate ratio of male and female categories in all collections). The per cent occurrences of different inferred age groups is shown in the figure for the sexed categories. Young-of-the-year squid below the size of seven

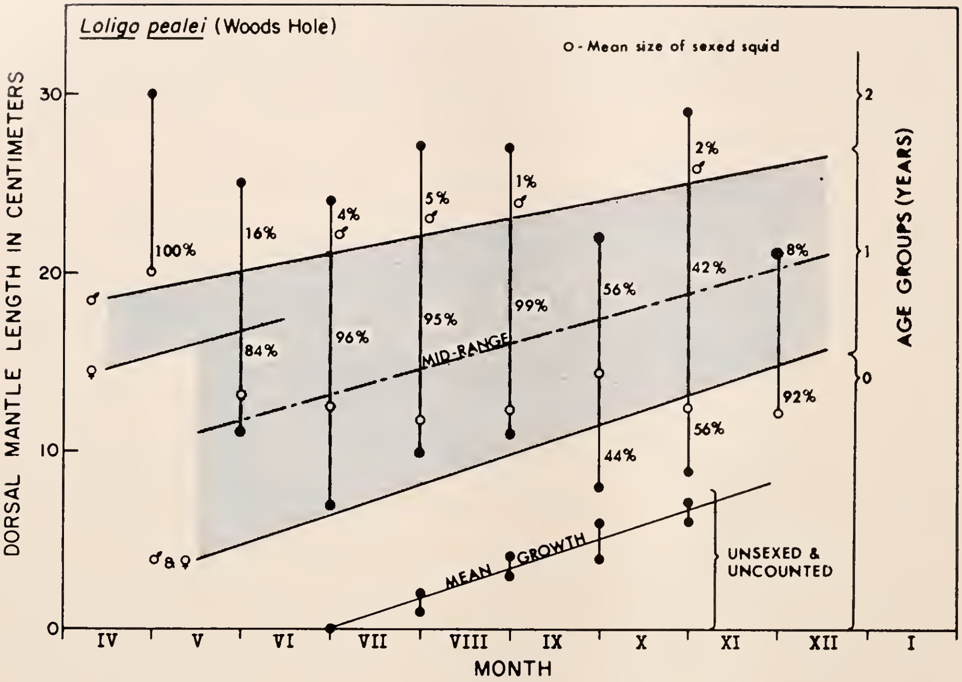


FIGURE 1. Monthly range of size class means, proposed age groups and occurrences of age groups near Woods Hole, Massachusetts. The data represent 8,437 squid from 45 collections in the years 1967–1970 and are a composite for males and females assuming a 1:1 sex ratio. The range of sizes for one year olds has been stippled for emphasis. Open dots indicate the mean size of all sexed squid (those with a dorsal mantle length of seven centimeters or larger). See text for further interpretation of the figure.

centimeters were specifically excluded from the quantitative results because they were generally a distinctive size class, their relative abundance was known to increase markedly over the inshore season (Summers, 1968) and because of probable escapement (reduced efficiency of the trawl net in collecting smaller squid).

Open dots in Figure 1 indicate the mean size of sexed squid; this value is remarkably constant after the middle of May and stays within the range 11½ to 14½ cm for seven months. The mean size can be misleading because it includes both sexes and varying proportions of different age groups. Sexual dimorphism in mantle length has been reported for *L. pealei* (Verrill, 1882; Haefner, 1964); our results indicate that males exceed the length of contemporary females by 0.5 cm at nine months, 2.0 cm at 12 months and 4–5 cm at 20 months. The mean size is useful in comparing unbiased samples of *L. pealei* with no segregation other than the exclusion of young-of-the-year individuals.

Three age groups are shown in Figure 1. One year olds (age group 1) are intermediate in size and age among these groups and most numerous among the sexed squid over the inshore season; the range of their size class means has been stippled in the figure for emphasis. Two year old squid arrived inshore near Woods Hole around the first of May. Two year old females were not taken after mid-June and only a minor proportion of two year old males were encountered after that date. One year olds arrived in large numbers in the latter half of May and remained to merge in size with the largest young-of-the-year squid by October. All two year old squid and almost all one year olds (into the month of August) were sexually mature and apparently breeding. Sexual maturity of one year olds decreased dramatically around the first of September. The majority of the young-of-the-year squid hatched around the first of July, these remained sexually immature and showed steady growth until the last squid migrated offshore in the latter part of November. Miscellaneous inshore samples were comparable to inshore trawl samples and no significant sample bias is indicated for sexed squid. In the winter collections, all female squid and most male squid below the size of 17 cm (*i.e.*, those less than two years old) were sexually immature (Summers, 1969).

DISCUSSION

Verrill's growth scheme for *L. pealei* can easily be imposed on the data presented in Figure 1. The broad range of sizes encompassing one year old squid can be readily identified and the absence of three or possibly four year old animals can be dismissed on the basis of rarity or possible avoidance of trawl nets. Verrill (1882) reported males of this species reaching a size of 42.5 cm, Summers (1968) noted a male 46.5 cm long and Vovk (personal communication) indicated that he had measured a male 45 cm long from offshore collections. Thus, larger (older) male squid exist in the population and the present data would seem to fall within Verrill's expansive life history model for *L. pealei*.

The size range indicated for one year olds (height of the stippled area in Figure 1) is particularly troublesome in applying Verrill's growth scheme. This range (approximately 13 cm) is conservative because it is a range of size class means and does not account for dispersion around those means. Given a continued growth of 1.8 cm per month, the rate observed for young-of-the-year squid (Summers, 1968), this range of sizes would have to result from a hatching period extending over at least seven months. A reduction in the growth rate over the first year would suggest a longer extrapolated hatching period. As noted above, mature female squid were encountered principally over a four month period; these included two age groups initiating breeding as much as one month out of phase with one another which produce a discrete brood near Woods Hole. Hence, the one year olds must represent a mixing from broods including some not observed near Woods Hole.

Reference to winter collections is instructive at this point. In March and April, *L. pealei* is concentrated near the continental shelf break and probably is compressed latitudinally from its summer range (Summers, 1969). Qualitative evaluation of the size distribution of winter squid clearly shows two size classes which, by extrapolated growth, would fall within the one year old size range given in Figure 1. These have modal sizes of approximately 8 and 14 cm dorsal mantle lengths. The smaller class was encountered in shallower depths from the southern mid-Atlantic Bight, it appeared to be the biological equivalent of the last young-of-the-year squid taken near Woods Hole, four months earlier. These I assume are a southern brood which probably hatches annually about the first week of

November. The larger class is identified clearly as the July brood shown in Figure 1. Cassie's technique did not indicate an intersection between these two broods at one year of age and the mid-range is tentatively used in Figure 1 to suggest their respective size ranges.

Data from the two broods have been synthesized and smoothed to produce the inferred growth scheme listed in Table I. Seasonal cycling of growth was not apparent in the data and none is indicated in the progression of listed mean sizes in the table. Sexual dimorphism can be calculated as the difference in mean sizes of contemporary male and female squid. The growth scheme suggests a decrease in monthly growth rate from 1.8 cm (young-of-the-year squid) to 1.1 cm for males and 0.9 cm for females over approximately a one and one-half year period. Based on winter samples (Summers, 1969), these data indicate weight doubling about every four months.

TABLE I

Growth scheme of Loligo pealei

Age (months)	Date, first of month		Mean dorsal mantle length (cm)	
	July brood	November brood	Females	Males
0	July	Nov.	0.2	0.2
2	Sept.	Jan.	4	4
4	Nov.	Mar.	7	7
6	Jan.	May	10	10
8	Mar.	July	12	12
10	May	Sept.	14	15
12	July	Nov.	16	18
14	Sept.	Jan.	18	21
16	Nov.	Mar.	20	23
18	Jan.	May	21	25
20	Mar.	July	23	28
22	May	Sept.	25*	30
24	July	Nov.	27*	32
.	.			.
.	.			.
.	.			.
34–36?	May–July	—	—	45

* Extrapolated from the observed data.

The two-brood scheme is especially useful in explaining biological observations such as the proximity of sizes of certain one year old and two year old squid, the relative constancy of mean sizes of sexed squid and the rapid change in sexual maturity of one year olds around September first. Furthermore, a number of species of squid are known to die soon after breeding, including the closely related *Loligo opalescens* from California (McGowan, 1954; Limbaugh and Shepard, 1957; Hobson, 1965; Fields, 1965). Data are lacking to demonstrate a breeding induced mortality in *L. pealei*, but should such a mortality exist, Verrill's growth scheme could not be reconciled with the observed breeding population.

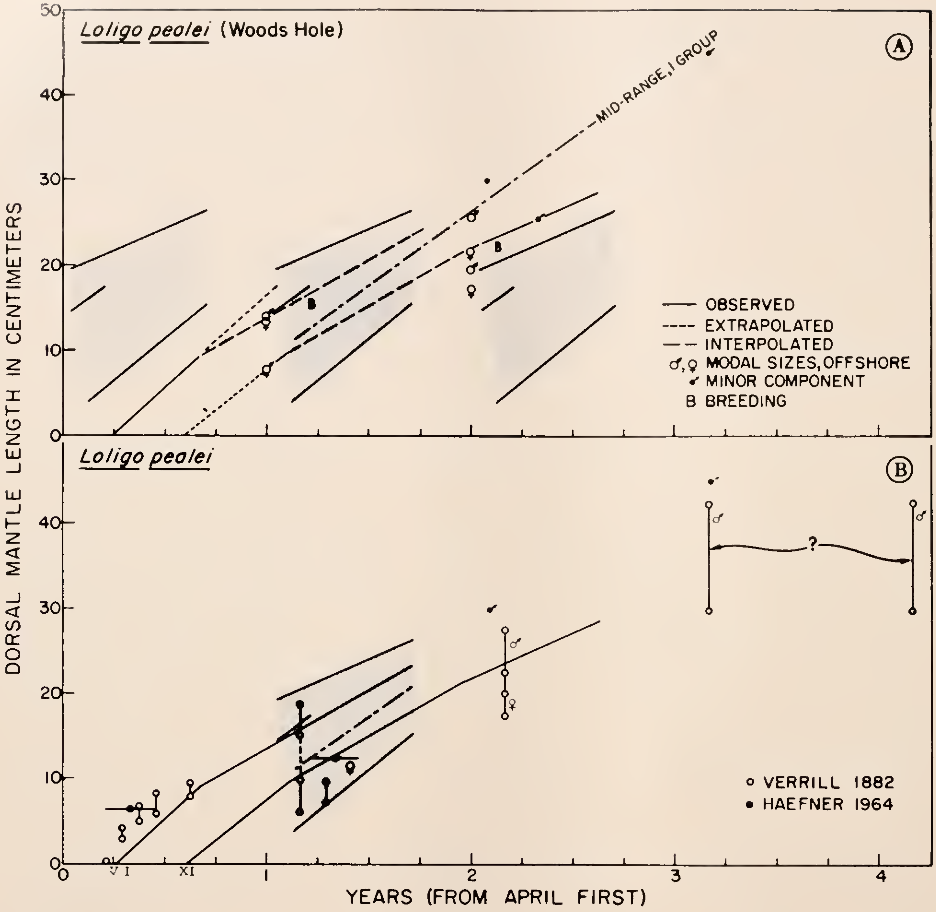
Counterparts of this growth scheme are not uncommon among other species of *Loligo*. The European squid, *Loligo vulgaris*, is reported to have a single hatching period in the North Sea during the month of June (Tinbergen and Verwey, 1945) and both June-July and fall hatches (if not year-round hatching) in the western Mediterranean (Mangold-Wirz, 1963). Fields (1965) reported a year-round hatch of *L. opalescens* is probable off California with a peak period in the month of May. In the southern hemisphere, *Loligo brasiliensis* is reported to breed from November through March (de Castellanos, 1967; de Castellanos *et al.,* 1968). Thus, squid of this genus tend to have a peak of breeding activity in the spring and, where temperatures are moderate, have a second breeding period in the fall with a tendency toward year-round breeding.

It follows that the size structure of *L. pealei* sampled near Woods Hole is composed of six elements. These are: young-of-the-year squid (July brood), one and two year olds (both broods each) and a very few three year old males (July brood?). The older squid migrate inshore in decreasing order of size and age, two year olds by May first and one year olds by June first. The July brood is represented among two year olds by a minor portion of the early arriving males, the remaining males and all of the females appear to be the product of the younger November brood. The July brood of one year olds is replaced by November squid beginning in August with the greatest transition taking place around September first as indicated by the decreasing proportion of sexually mature animals. In November, many of the one year old males begin to exhibit a white coloration of the vas deferens which portends developing sexual maturity.

Older groups are probably replaced by serial inshore migrations and mortality after breeding. The occurrence of males 22 to 24 months old (and a very few as much as three years old) when females do not ordinarily live 20 months suggests that males may be less subject to breeding mortality. The net survival advantage of males cannot be too great, because imbalanced sex ratios were not observed in the sampling. This paradox could result from competition for females leading to postponed breeding among the males. Arnold (1962) reported that the largest male squid were the first to breed under laboratory conditions.

Mortality of *L. pealei* is difficult to estimate because all age groups cannot be adequately sampled at any one place or time. We have not detected strong year classes in our brief sampling and have regularly noted an inverse relationship between age and abundance. Sexually mature female squid bear between 3500 and 6000 eggs (depending on their size) which appear to approach 100% hatch in nature. For the population to remain stable, these eggs must replace two individuals if both sexes breed only once in a lifetime and there is no net recruitment. Under these assumptions, the annual survival of the June breeding population is approximately one in two thousand at its lower level.

A positive skew in the size distribution of young-of-the-year squid sampled near Woods Hole allows some refinement in the mortality estimate. The skew cannot be related to temperature lability in embryonic development (McMahon and Summers, unpublished). A lognormal model readily fits the observed distribution (Summers, 1968). (The same model seems appropriate for *L. brasiliensis* as reported by de Castellanos, 1967; de Castellanos *et al.*, 1968.) The tail of the observed size distribution must result from early breeding, probably by two year old squid. As shown in Figure 1, all of the *L. pealei* taken in the month centering on the first of May and one-sixth of those in the following



month were two year olds. Perhaps one-fourth of the egg deposition around June first was carried out by two year old females. Approximately 10% of the brood hatching ahead of the normal distribution would produce the skew size distribution. Thus, two year olds (mostly 18 and 19 months old) probably account for less than one-quarter of the total breeding population and contribute no more than one-third of the brood.

Interbreeding between age groups is likely. In the laboratory, older males frequently breed with younger females and the natural age structure suggests the same is true in the field. Interbreeding of this sort should have no net effect on the proportions stated above. Unfortunately, these data cannot be used to estimate the survival from 11 months of age to 19 months because different broods are involved and recruitment is apparent for at least one of them. It is consistent to suggest that *L. pealei* is mainly annual and that mortality follows breeding in both sexes. Sexual maturity is deferred in some members of the November brood and sexual regression need not occur.

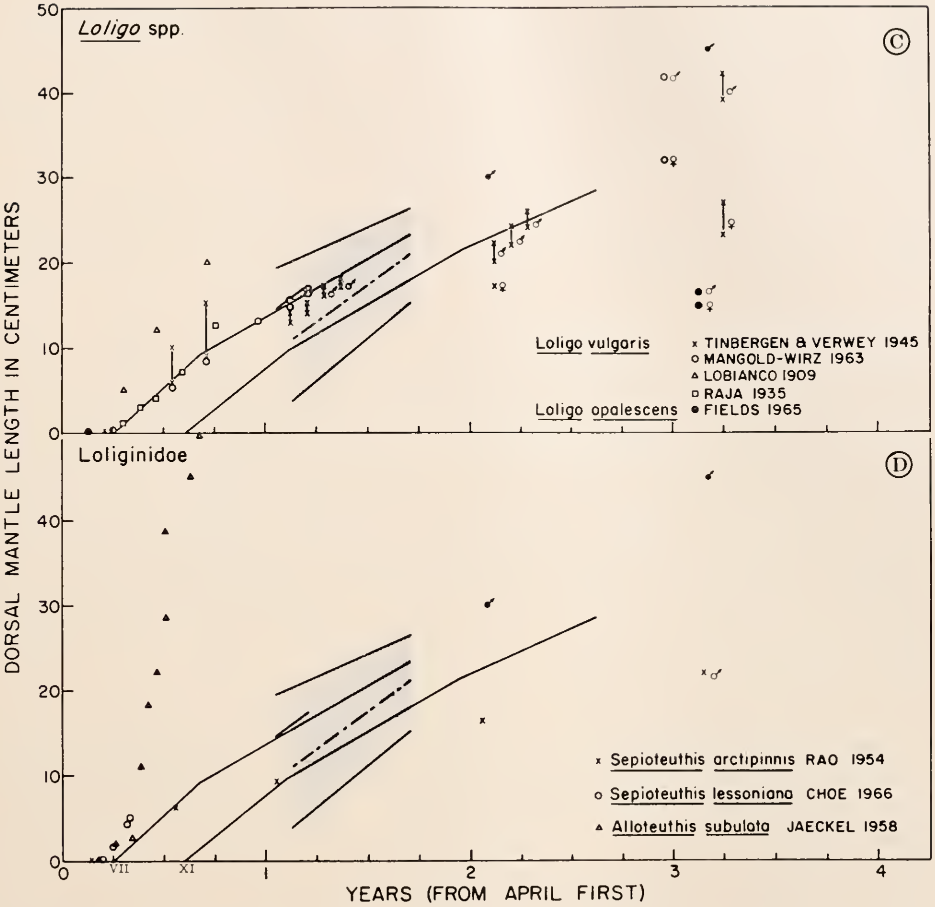


FIGURE 2. Shown in Figure 2A is a summary of the proposed growth scheme for *L. pealei* representing data from 15,132 squid. The range of sizes for one year olds (stippled areas) and mean growth of young-of-the-year squid are reiterated from Figure 1. A skeletal abstract of Figure 2A is repeated in Figures 2B–2D with data from the literature; these are arranged by taxa as indicated. Tinbergen and Verwey (1945) reported ventral mantle lengths which are slightly shorter than corresponding dorsal mantle lengths.

The growth scheme presented here can be used to anticipate latitudinal differences in age structure. One can postulate that two year olds (or older squid) would be rare or absent in areas where a significant November hatch occurs, especially south of Hudson Canyon. This tendency should be reflected by truncated ranges of size class means and lower mean sizes of sexed squid when compared to the data shown in Figure 1. The northern range limit of *L. pealei* has been recently described as coastal, southern Nova Scotia and the Bay of Fundy (Mercer, 1970). Squid taken as far north as the Bay of Fundy probably reflect selection for the capacity to make a long migration from the wintering grounds (Summers, 1969) and may show higher mean sizes. Stevenson (1934) observed the occurrence of *L. pealei* at St. Andrews, New Brunswick (Canada) "early in the summer" of 1932. He reported a peak abundance in a weir about mid-August and breeding activity in late August. Dr. and Mrs. Kay Petersen (personal communication) found egg masses and at least one newly hatched *L. pealei* in Minas Basin, Bay of Fundy late in the summer of 1970. These spotty data suggest that the "July brood" is delayed that far north.

A summary of the inferred growth scheme, winter data and major features of the inshore collections are shown in Figure 2A. Except as indicated in the figure, the information applies to a statistical intersex squid. A skeletal abstract of Figure 2A is repeated in Figures 2B, 2C and 2D for comparison with data from the literature on different genera and species of squid. Verrill's (1882) tabulated size ranges are shown in Figure 2B. Verrill listed a split range of sizes for one year olds which corresponds with the two broods suggested here. The simplicity of his one brood growth scheme is apparent in the figure. Haefner (1964) pooled the size distribution of squid collected over the period June 10 to September 17, 1958 in Delaware Bay; modal sizes from these data are shown in Figure 2B. Haefner's squid (like Verrill's) appear to have hatched earlier than those sampled recently near Woods Hole. His most distinctive older size class was a group of females which I would identify as the previous November brood (7 to 10 months old in his samples). Not shown in Figure 2B is yet another interpretation of the growth of *L. pealei* by Jaeckel (1958), who suggested an arbitrary reduction in the assigned age of Verrill's older groups. This modification is not supported by original data, but suggests that a higher growth rate might be appropriate.

As shown in Figure 2C, data describing the growth of *L. vulgaris* can also be fitted by the growth scheme presented here for *L. pealei*. Exceptions include Lo Bianco's (1909) data and the three year old females in Tinbergen and Verwey's (1945) report. Most recent authors have noted similarity in life history aspects between *L. pealei* and *L. vulgaris* and this certainly extends to their age and growth.

Fields (1965) indicated a uniform growth rate for *L. opalescens*, the end points of which are shown in Figure 2C. In view of the reports cited above, it is likely that Fields overestimated the age of *L. opalescens* and that he was dealing with an annual population.

Data for three other loliginid squid are shown in Figure 2D. It should be noted that *Alloteuthis subulata* exhibits an exceptionally long mantle length for its body proportions due to the development of a pointed projection on the mantle. None of the data shown in Figure 2D offer a useful contrast with the growth scheme of *L. pealei*.

Growth models for three species of *Loligo* can be grouped in four types: linear (*L. opalescens*, Fields, 1965); cyclic (*L. vulgaris*, Tinbergen and Verwey, 1945); von Bertalanffy (*L. pealei*, Verrill, 1882) and non-asymptotic (*L. vulgaris*, Mangold-Wirz, 1963; *L. pealei*, Summers, this report). The first of these is useful over short increments of time or in the absence of more complete information because it probably does not correctly describe the growth. Cyclic models are difficult to compare and lead to speculation about the effects of migration or selective mortality in the sampled population. The absence of reliable age markings in squid and apparent continued growth through cold seasons tend to rule out growth cycles. The last two models differ mainly in their degree of non-linearity and I have arbitrarily separated them on a basis of a factor of two in the growth rate. The von Bertalanffy (1934) growth model shows a wide change in instantaneous growth rates and indicates an approach to an asymptotic size during the animal's life span. Non-asymptotic models suggest that the oldest animals are still actively growing as is generally true for mollusks (Van Cleave, 1934; Russell Hunter, 1961). The applicability of these various models would be most convincingly demonstrated through studies of individual squid, especially by the results of tagging studies or prolonged laboratory maintenance.

The author wishes to acknowledge the assistance of many individuals who have contributed to this study in various ways from 1966 to the present time. Some of these have been cited in earlier papers as appropriate in specific phases of the work. I am indebted to my assistants, Miss Paula Mogilni, Mr. Steven Slomka, Mr. Michael Soukup and Mr. John McMahon, for their patience and hard work. I especially appreciate the stimulation, support and guidance provided by Drs. H. Burr Steinbach, Daniel L. Gilbert and John M. Arnold, all of the Marine Biological Laboratory. Finally, I wish to thank Mr. McMahon and Drs. Leland W. Pollock and Melbourne R. Carriker who read and criticized the manuscript.

SUMMARY

1. This paper describes the population size structure, inferred age, growth, reproduction and longevity of the common Atlantic Coast squid, *Loligo pealei.*

2. The sampling includes records of size (dorsal mantle length), sex and sexual maturity of 15,132 squid taken from 1967–1970. Nearly half of these were collected offshore between Cape Hatteras and Georges Bank in the late winters of 1967 and 1968. The remainder, including planktonic, young-of-the-year squid, were trawled in the vicinity of Woods Hole, Massachusetts between May and November of all three years.

3. Size classes were identified and weighted through the use of size frequency analysis and arrayed to provide an empirical growth model. Mean sizes of individuals appeared to increase smoothly to 16 and 18 cm at one year and 27 and 32 cm at two years for females and males, respectively.

4. Two broods arise each year, a ubiquitous July brood (probably delayed north of Cape Cod) and a November brood which probably originates in the southern mid-Atlantic Bight. Sexual maturity and breeding have not been observed at less than one year of age; at Woods Hole these features occur at different ages and slightly different dates for the two broods. Competition for females may postpone the breeding of some males and exaggerate the population sexual dimorphism.

5. The stock is basically annual, though a significant proportion of the squid hatching near Woods Hole appear to be the product of two year olds. A breeeding induced mortality is consistent with the growth scheme for both sexes.

This mechanism is evoked to explain the dynamics of age structure and sexual maturity during the inshore season.

6. Maximum longevity is understood tentatively to be 36 months for males (more frequently 20–24 months) and 19 months for females. Sex ratios were consistently close to 1:1 though not necessarily balanced in older age groups.

7. The proposed growth scheme provides an hypothesis for latitudinal variations in the stock of *L. pealei.*

8. The results are compared with Verrill's influential treatment of the species and found to differ principally in the interpretation of data. The proposed growth scheme appears to be applicable to published data for the European squid, *L. vulgaris,* and is contrasted with records from other loliginid squid.

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Continued on Cover Three

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THE
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THE TUBIFICIDAE (ANNELIDA, OLIGOCHAETA) OF CAPE COD BAY. II: ECOLOGY AND SYSTEMATICS, WITH THE DESCRIPTION OF *PHALLODRILUS PARVIATRIATUS* NOV. SP.[1]

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Woods Hole, Massachusetts 02543

The systematics and ecology of marine organisms in Cape Cod Bay are being studied by means of year-round, qualitative and quantitative benthic samples, collected by the Biotic Census which was initiated by the Systematics-Ecology Program (SEP), Marine Biological Laboratory, Woods Hole, Massachusetts. This account is the second of a series which will report on the marine tubificid Oligochaeta of Cape Cod Bay.

Quantitative samples were taken at one mile intervals over the entire bay. The sampling pattern is based on a system of 1 mile square quadrats, identified by four digit numbers (Fig. 2). Samples were taken by a Smith-McIntyre grab (0.10 m^2) at the center (E1) and at each corner (E2 to E5 running clock-wise from E2 in the northeast corner) of every alternate quadrat in the north-south direction. The samples were separated into two fractions by 1.0 mm and 0.5 mm mesh screens and the material narcotized in 0.015% propylene phenoxetol, fixed in 10% formalin (for 48 hours) and stored in 85% ethanol (McKay and Hartzband, 1970). Qualitative samples, treated in the same manner as the quantitative material, were taken from three corners of the quadrats (see Table I and II for exact locations) by epibenthic sled (Ep), clam dredge (C), and naturalist dredge (N). Oligochaeta from 210 sorted 1.0 mm screen fractions have been examined.

The systematics of five of the species of Tubificidae found in Cape Cod Bay (*Peloscolex intermedius* Cook, 1969, *Adelodrilus anisosetosus* Cook, 1969, *Phallodrilus coeloprostatus* Cook, 1969, *Phallodrilus obscurus* Cook, 1969 and *Limnodriloides medioporus* Cook, 1969) and methods of their examination have been dealt with elsewhere (Cook, 1969). In the systematic section of the present account, the ten species found in the bay are listed with the station numbers, including abundance and physical data, at which they occurred. An additional species, *Phallodrilus parviatriatus* nov. sp. is described, *Peloscolex apectinatus* Brinkhurst, 1965 and *Peloscolex nerthoides* Brinkhurst, 1965, are added to the original list,

---

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and the description of *Tubifex longipenis* Brinkhurst, 1965, previously known from a single mature individual, is confirmed and expanded. The ecological section attempts to explain the distribution of the species in relation to the particle size of the substrate.

SYSTEMATICS AND DISTRIBUTION

Adelodrilus anisosetosus Cook, 1969

Adelodrilus anisosetosus Cook, 1969, pages 13–15, Figure 3.

DISTRIBUTION: see Table I (A.2).

REMARKS: This species is known only from Cape Cod Bay. Breeding individuals were found in January, May, June and November but on the slender data available it is impossible to generalize on the life-history of the species.

Phallodrilus obscurus Cook, 1969

Phallodrilus obscurus Cook, 1969, pages 17–18, Figure 6.

DISTRIBUTION: Stations 1730-E3 (2 individuals) and 2130-E2 (2 individuals).

REMARKS: Known only from Cape Cod Bay. Breeding individuals were found in November only.

Phallodrilus coeloprostatus Cook, 1969

Phallodrilus coeloprostatus Cook, 1969, pages 16–17, Figure 5.

DISTRIBUTION: see Table I (Ph.1).

REMARKS: Known only from Cape Cod Bay. Breeding individuals were found in January, April, May, June, August, September and November, and it would seem that *P. coeloprostatus* is capable of reproduction at any season of the year.

Phallodrilus parviatriatus nov. sp.

Figure 1

HOLOTYPE: United States National Museum (USNM) Cat. No. 42015. Cape Cod Bay, Massachusetts, U. S. A. 41° 54.0′ N, 70° 8.6′ W. Depth 17.1 meters. Collected June 11, 1968. (SEP Station number 1412-E4).

PARATYPES: USNM 42016. Two individuals, data as for holotype. USNM 42017. Five individuals, 41° 53.5′ N, 70° 10.7′ W. Depth 18.0 m. Collected January 19, 1967. (SEP Sta. No. 1514-E1). National Museum of Natural Sciences, Ottawa, Canada, Cat. No. 3413. One individual, data as for holotype.

ETYMOLOGY: "parvus" = L. "small"; hence "having a small atrium."

DESCRIPTION: About 9.5 mm long, 0.3 to 0.4 mm diameter anteriorly, 0.48 mm diameter at segment XI, and 0.35 to 0.43 mm posteriorly. Approximately 64 segments. Prostomium rounded, longer than it is wide at peristomium junction.

Clitellum developed on segments X to XII, but only weakly so, or absent, on ventral surface of segment XI. Dorsal and ventral setae similar in number, size and shape: anteriorly each setal bundle contains 3 to 5 bifid setae, 62 to 87 μ long

TABLE I

Distribution of coarse-sand-dwelling Tubificidae: Ph.1 = Phallodrilus coeloprostatus; *A.2* = Adelodrilus anisosetosus; *T.3* = Tubifex longipenis; *P.4* = Peloscolex benedeni; *P.5* = Peloscolex apectinatus; *— = absent; + = present; ? = no data;* ϕ *=* $\log_2$ *particle diameter in mm*

Station number	No. specimens in 1 mm screen fraction (E1–E5) per 0.10 m²					Date	Depth (m)	Median ϕ
	Ph.1	A.2	T.3	P.4	P.5			
0612-E1	2	—	—	30	1	4/24/68	11.0	0.20
0612-E5	—	—	—	1	—	4/24/68	4.3	?
0616-E1	6	—	2	4	—	8/18/69	7.0	0.27
0616-E4	15	—	11	2	—	8/18/69	51.2	?
0616-E5	38	—	24	6	—	8/18/69	8.2	?
0714-E4	—	—	—	32	—	10/16/69	42.4	−0.80
1110-E1	—	—	30	—	—	8/19/69	5.8	0.73
1212-E1	—	—	—	18	—	3/23/69	21.1	0.25
1212-N (at E3)	—	—	—	+	—	3/23/69	16.2	?
1412-E1	40	—	7	82	—	6/11/68	15.6	−0.57
1412-E2	2	—	21	8	—	6/11/68	13.4	?
1412-E3	—	4	232	—	—	6/11/68	11.6	?
1412-E4	67	2	37	796	1	6/11/68	17.1	0.35
1412-E5	—	—	2	80	—	6/11/68	21.1	−0.27
1412-Ep (E4)	—	—	—	+	—	6/11/68	17.1	?
1412-N (E2)	—	—	—	+	—	6/11/68	13.4	?
1510-E1	3	—	—	—	—	9/11/69	3.4	1.37
1514-E1	3	—	9	135	—	1/19/67	18.0	−0.34
1514-E2	—	1	—	35	1	1/19/67	18.3	−0.28
1514-E3	—	—	41	10	—	1/19/67	18.3	?
1514-E4	—	—	2	42	—	1/19/67	18.3	−0.20
1514-N (E4)	—	—	—	+	—	1/19/67	18.3	?
1514-C (E2)	+	—	+	+	—	1/19/67	18.3	?
1530-N (E2)	—	—	—	—	+	11/21/67	11.3	1.83
1612-E1	43	—	158	33	43	5/13/69	8.5	0.48
1714-E4	—	26	25	3	1	1/21/69	18.6	?
1714-C (E3)	—	—	+	+	—	1/21/69	13.7	?
1730-E3	—	—	—	—	9	11/21/67	8.5	0.81
1816-E1	25	8	—	59	20	5/13/69	21.1	0.26
1816-C (E2)	—	—	—	—	+	5/13/69	20.8	?
1910-E1	—	—	2	1	—	5/17/67	6.7	−0.03
1910-E3	—	—	1	—	—	5/17/67	6.7	0.97
1910-N (E3)	—	—	+	—	—	5/17/67	6.7	?
1914-E1	—	—	42	9	—	11/18/68	11.9	0.43
1930-E2	3	10	—	—	—	11/21/67	10.4	0.54
2016-E3	3	—	—	—	—	6/12/68	12.8	?
2016-E4	—	—	—	2	—	6/12/68	18.0	?
2028-E5	—	—	—	—	2	3/21/66	18.3	?
2110-E1	—	—	8	—	—	9/11/69	7.6	0.61
2130-E2	—	—	22	—	—	11/21/67	6.7	1.12
2212-N (E4)	—	—	+	—	—	4/23/68	6.1	?
2318-E4	—	—	—	10	—	10/13/66	15.0	−0.74

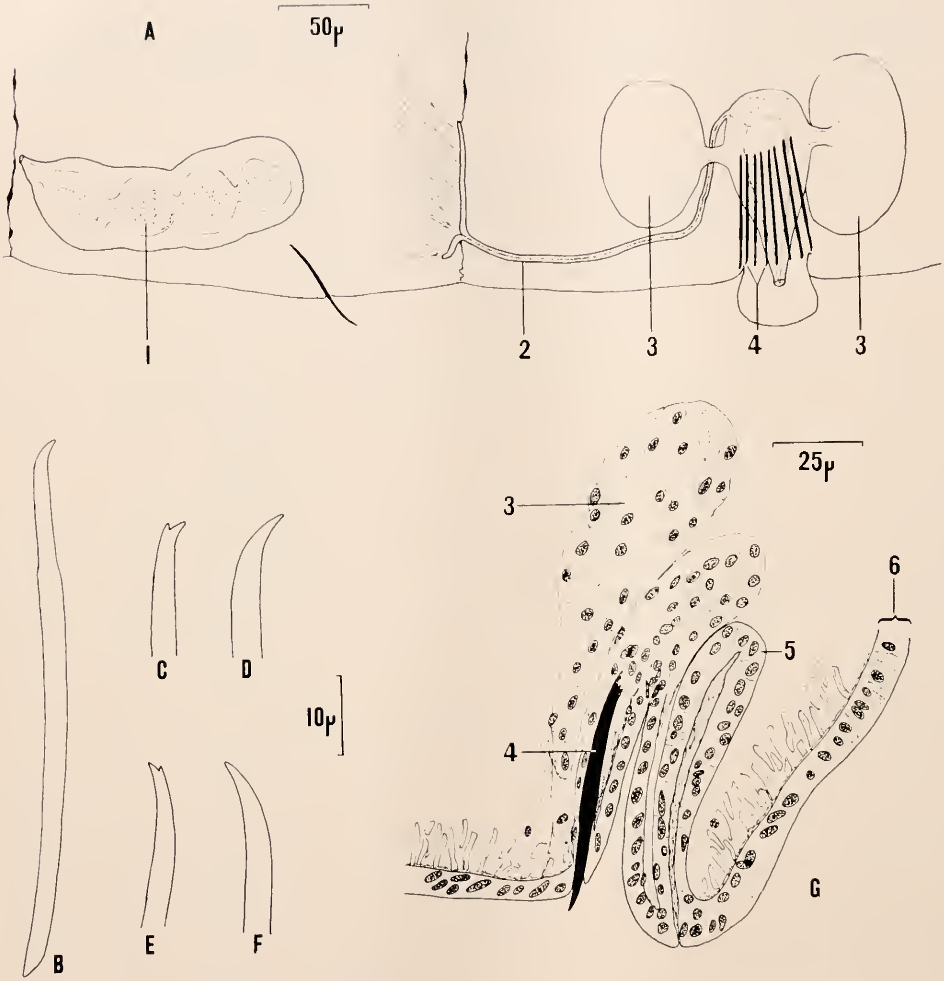

Figure 1. *Phallodrilus parviatriatus* nov. sp.; (a.) Lateral view of the genital segments (compiled from dissections); (b.) Penial seta; (c.) Dorsal seta from segment VII; (d.) Dorsal seta from segment XIII; (e.) Ventral seta from segment III; (f.) Ventral seta from segment XII; (g.) Transverse section through the male pore and atrium (USNM 42016): (1.) Spermatheca; (2.) Vas deferens; (3.) Prostate gland; (4.) Penial seta; (5.) Atrium; (6.) Body wall epithelium.

(Fig. 1c and e); posteriorly each bundle contains 2 to 3 simple-pointed setae, 62 to 83 µ long (Fig. 1d and f). Setae bifid with upper teeth shorter than the lower, up to about segment VIII, reaching their maximum length in the region of segments VIII to XV. Ventral setae of segment XI modified into penial bundles each containing 7 to 10 single-pointed setae 75 to 88 µ long (Fig. 1b). Very small, paired, spermathecal pores located at the lateral line of intersegmental furrow IX/X. Paired male pores open on the summits of paired ventro-lateral protuberances just lateral to penial setae.

Coelomocytes small and few in number (but in one paratype from 1514-E4, coelomocytes up to 15 μ diameter were distributed thus: 7 or 8 dorsal in segment VII, 5 dorsal and a clump of 15 to 20 ventrally in segment VIII, 15 to 20 ventral in segment IX, and 7 dorsal in segment X). Pharyngeal glands present up to segment V. Chlorogogen cells begin in segment V. Male genital system (all structures paired—Fig. 1a): relatively large male funnel opens into vas deferens (200 to 220 μ long, 7 to 11 μ diameter in holotype) which runs along the ventral side of segment XI then turns dorsally to join the atrium subapically (antero-dorsally). Atrium erect, elongate pear-shaped, laterally flattened; atrium 84 to 100 μ long and consists of a single layer of cells 5 to 10 μ thick (Fig. 1g); external width of atrium (anterior-posterior direction) 40 to 58 μ, and 26 to 30 μ (lateral direction). Two pear-shaped prostate glands, about 125 μ long, 62 μ wide, join each atrium by thick discrete ducts, one anterior, the other posterior. Paired spermathecae, 133 to 160 μ long 45 to 70 μ wide, situated laterally in the anterior part of segment X: ampullae, which are constricted at intervals giving the appearance of a string of closely-joined spheres, terminate in very small conical ducts; latter open directly to the exterior (*i.e.*, discrete spermathecal ducts absent). Sperm in spermathecae in loose random masses. Median, unpaired, sperm sac extends anteriorly to segment VIII and posteriorly to about segment XIV.

DISTRIBUTION: Stations 1412-E4 (50 individuals); 1514-E1 (3); 1514-E3 (1); 1514-E4 (4).

REMARKS: Known only from Cape Cod Bay. Breeding individuals were found in January and June. *P. parviatriatus* is easily distinguished from other members of the genus by its small erect atria and simple-pointed posterior setae (see Cook, 1969).

Tubifex longipenis Brinkhurst, 1965

Tubifex longipennis Brinkhurst, 1965, page 124, Figure 2j–l. (Typographical error in original.)

Tubifex longipenis. Brinkhurst and Cook, 1966, page 14: Cook, 1969, page 10.

HOLOTYPE: USNM 32605. Five Islands, Dry Point, Georgetown, Maine, U. S. A.

ADDITIONAL MATERIAL: Gray Museum, Marine Biological Laboratory, Woods Hole.

DESCRIPTION: Length 25 to 30 mm, diameter 0.43 to 0.53 mm anteriorly, 0.27 mm posteriorly. Number of segments, 85 to 108. Dorsal and ventral setae similar in number, size and shape: anteriorly each setal bundle contains 2 to 4 broad bifid setae, 110 to 130 μ long, with the upper teeth shorter than the lower; posteriorly, from about segment XII, each bundle contains 1 simple-pointed seta, 110 to 140 μ long, 6.5 to 8.5 μ thick. No modified genital setae.

Pharyngeal glands present in segments IV and V. Atria elongate, tubular, 440 to 510 μ long, 45 to 150 μ diameter, penetrate posteriorly into segment XII. Vasa deferentia, 20 to 23 μ diameter, very long and coiled, join the atria dorsally opposite to the prostate gland opening. Atria terminate in elongate, thickly-cuticularized penes, 320 to 450 μ long, 34 to 73 μ diameter, each of which bears a cuticu-

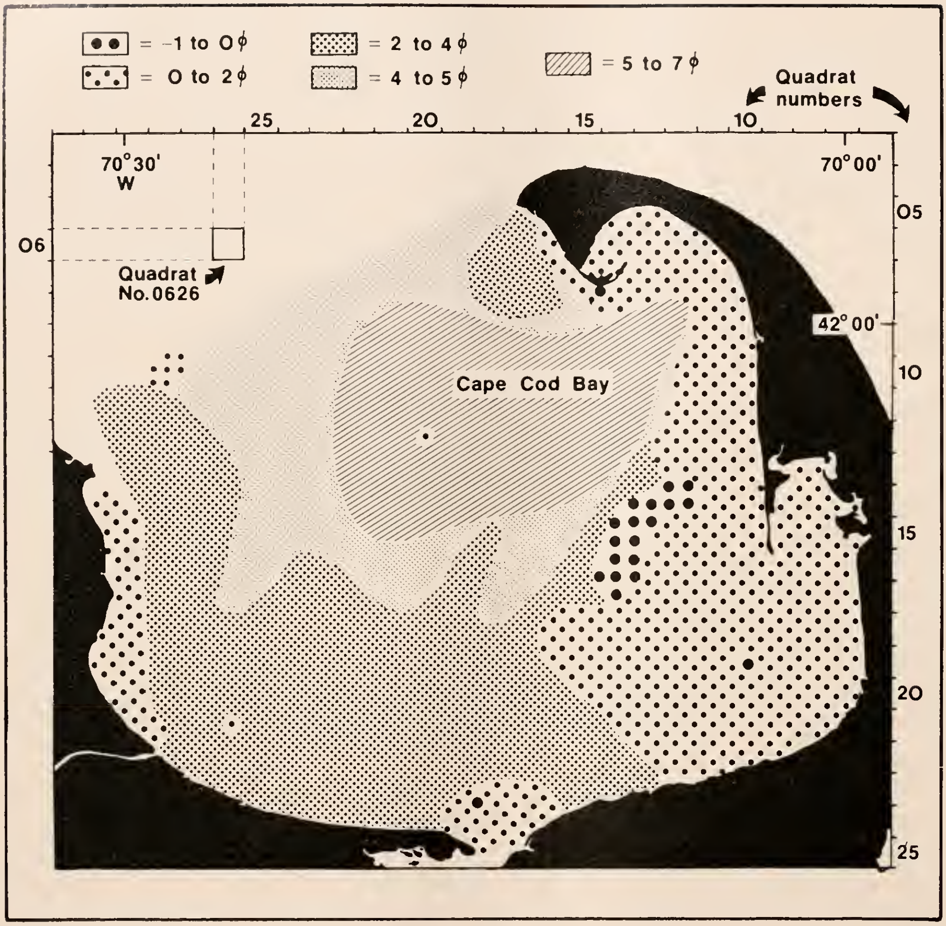

Figure 2. Sediment distribution, expressed in terms of median particle size (ϕ units = $\log_2$ particle diameter in mm), in Cape Cod Bay. Sediment analyses were performed mainly on E1 samples, hence the sediment boundries shown may contain errors of about 2 miles in any direction. The marginal figures indicate the system used for quadrat identification.

larized hook about 30 μ long, near its distal end. Paired spermathecae with discrete ducts and sub-spherical ampullae, open just anterior to ventral setae of segment X.

Distribution: see Table I (T.3) for Cape Cod Bay; Georgetown, Maine.

Remarks: The original description (Brinkhurst, 1965) was based on a single specimen. The above description is based on the holotype and additional material from Cape Cod Bay which confirmed the peculiar hooked nature of the penis sheath. Breeding individuals of *T. longipenis* were found in August and September only, and cocoons were present in a January sample which suggests that breeding probably occurs in fall, the cocoons overwinter and hatch the following spring.

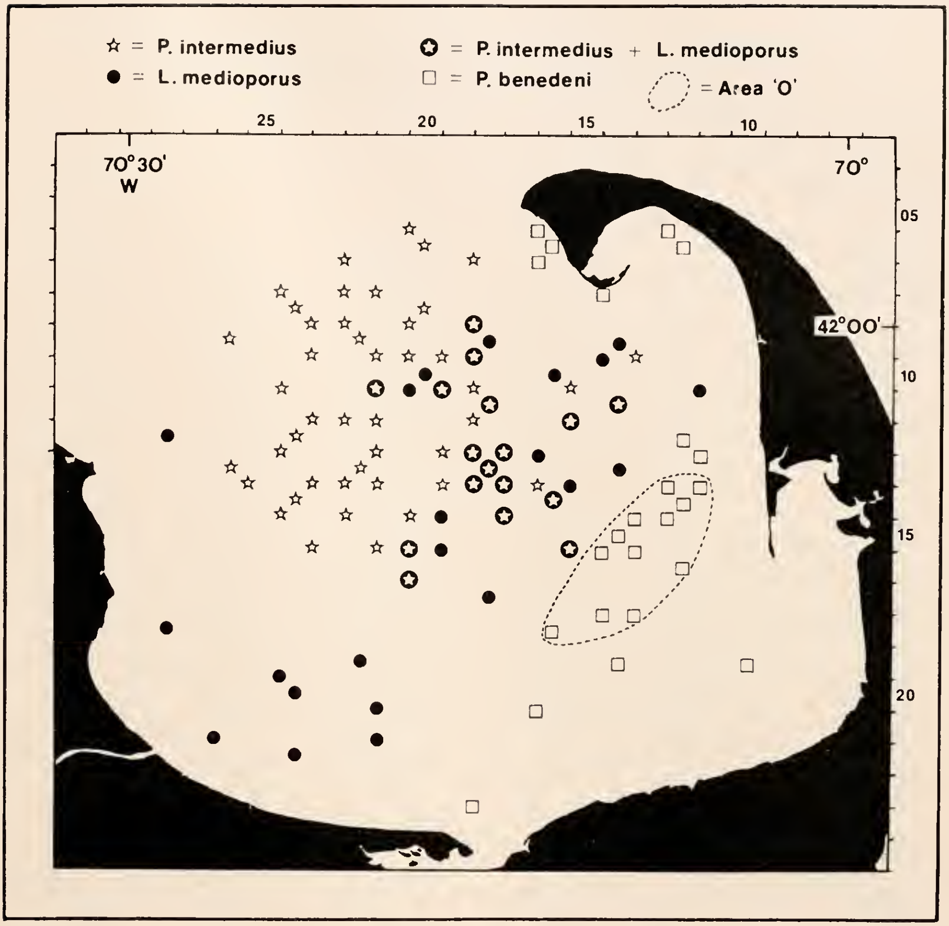
☆ = P. intermedius
● = L. medioporus
✪ = P. intermedius + L. medioporus
□ = P. benedeni
= Area 'O'
25
20
15
10
70°30' W
70°
05
42°00'
10
15
20

Figure 3. Distribution of *Peloscolex intermedius*, *P. benedeni* and *Limnodriloides medioporus* in Cape Cod Bay. The area of maximum species concentration (area "O") is indicated by the dotted line.

Peloscolex benedeni (Udekem, 1855)

Tubifex benedeni Udekem, 1855, page 544.
Peloscolex benedeni (Udekem). Brinkhurst, 1965, page 133; Cook, 1969, page 11. (For full synonomy see Brinkhurst and Jamieson, 1971.)

Distribution: see Table I (P.4) and Figure 3 for Cape Cod Bay; Europe; Atlantic coast of North America. A widely distributed species in both brackish-water and fully marine habitats.

Peloscolex apectinatus Brinkhurst, 1965

Peloscolex gabriellae var. *heterochaetus* Brinkhurst, 1965, page 133. (Typographical error in original.)

TABLE II

Distribution of fine-sand and silt-dwelling Tubificidae: P.6 = Peloscolex intermedius; *L.7* = Limnodriloides medioporus; *—* = *absent;* + = *present;* *?* = *no data;* ϕ = $\log_2$ *particle diameter in mm*

Station number	No. specimens per 0.10 m^2 (1 mm)		Date	Depth (m)	Median ϕ
	P.6	L.7			
0620-E1	1	—	4/18/67	56.5	4.72
0620-E5	1	—	4/18/67	59.5	?
0620-Ep (E5)	+	—	4/18/67	59.5	?
0718-Ep (E5)	+	—	1/6/69	57.0	?
0722-Ep (E5)	+	—	1/20/69	54.6	?
0722-N (E4)	+	—	1/20/69	53.1	?
0722-C (E3)	+	—	1/20/69	54.3	?
0820-E1	1	—	8/20/68	50.9	5.97
0820-N (E4)	+	—	8/20/68	50.4	?
0824-E1	4	—	6/28/67	49.1	4.63
0824-E3	1	—	6/28/67	49.1	?
0824-E5	1	—	6/28/67	50.0	?
0824-Ep (E3)	+	—	6/28/67	49.1	?
0824-N (E5)	+	—	6/28/67	50.0	?
0914-E1	—	1	2/20/68	31.4	5.95
0914-E4	—	1	2/20/68	33.8	?
0914-N (E3)	+	—	2/20/68	35.1	?
0918-E1	—	70	1/23/68	41.8	5.65
0918-E4	4	5	1/23/68	42.1	?
0918-E5	4	4	1/23/68	45.4	?
0922-E1	8	—	3/27/68	46.4	5.95
0922-E5	2	—	3/27/68	47.6	?
0922-C (E3)	+	—	3/27/68	44.5	?
0926-E1	1	—	6/10/68	47.3	4.17
1012-E3	—	4	9/7/67	21.6	?
1016-E1	—	8	8/19/68	36.0	6.20
1016-N (E3)	+	—	8/19/68	34.2	?
1020-E1	—	1	3/23/67	45.8	6.15
1020-E2	1	—	3/23/67	43.3	?
1020-E3	29	9	3/23/67	42.4	?
1020-E4	—	1	3/23/67	42.7	?
1020-E5	2	—	3/23/67	45.8	?
1020-Ep (E5)	+	—	3/23/67	45.8	?
1020-N (E3)	+	—	3/23/67	42.4	?
1024-E4	16	—	12/19/68	44.5	?
1024-Ep (E2)	+	—	12/29/68	48.5	?
1114-E1	1	19	3/11/69	31.7	5.41
1118-E1	10	8	9/30/68	42.7	6.33
1118-Ep (E4)	+	—	9/30/68	42.4	?
1118-C (E5)	+	—	9/30/68	42.4	?
1122-Ep (E4)	+	—	12/19/68	44.5	?
1122-N (E3)	+	—	12/19/68	43.6	?
1122-C (E2)	+	+	12/19/68	47.3	?
1216-E2	—	4	12/19/67	32.3	?
1216-E4	—	22	12/19/67	32.3	5.82
1216-N (E2)	+	—	12/19/67	32.3	?
1220-E3	3	—	8/26/68	36.9	?
1224-E1	1	—	3/27/68	42.7	4.63

TABLE II—(*Continued*)

Station number	No. specimens per 0.10 m² (1 mm)		Date	Depth (m)	Median ϕ
	P.6	L.7			
1224-Ep (E4)	+	—	3/27/68	40.2	?
1224-N (E2)	+	—	3/27/68	43.9	?
1228-E1	—	1	5/10/67	33.2	3.38
1314-E1	—	206	4/23/68	29.9	5.81
1318-E1	1	5	6/14/67	35.7	5.72
1318-E2	2	9	6/14/67	36.6	?
1318-E3	16	42	6/14/67	36.0	?
1318-E4	9	6	6/14/67	35.1	?
1318-E5	43	3	6/14/67	38.4	?
1318-N (E2)	+	—	6/14/67	36.6	?
1322-E1	18	—	7/24/67	36.3	5.31
1322-E2	14	—	7/24/67	40.3	?
1322-E3	3	—	7/24/67	37.5	?
1322-E4	13	—	7/24/67	38.4	?
1322-N (E2)	+	—	7/24/67	40.3	?
1326-E1	1	—	7/22/66	35.7	3.63
1326-Ep (E3)	+	—	7/22/66	36.6	?
1416-E1	1	29	10/1/68	32.0	5.50
1416-Ep (E5)	+	—	10/1/68	32.6	?
1416-C (E2)	—	+	10/1/68	32.0	?
1420-Ep (E2)	+	—	12/12/68	33.9	?
1420-N (E4)	+	—	12/12/68	33.3	?
1420-C (E3)	—	+	12/12/68	32.9	?
1424-E1	1	—	11/19/68	38.1	4.73
1424-E4	1	—	11/19/68	39.4	?
1424-Ep (E2)	+	—	11/19/68	39.4	?
1424-N (E4)	+	—	11/19/68	36.9	?
1518-E2	2	2	1/23/68	34.2	?
1518-N (E2)	+	—	1/23/68	34.2	?
1522-Ep (E5)	+	—	8/19/68	33.2	?
1522-N (E3)	+	—	8/19/68	31.7	?
1616-E2	3	37	4/22/68	28.7	?
1620-E2	—	2	12/19/67	33.2	?
1620-E4	—	1	12/19/67	32.3	?
1620-E5	3	2	12/19/67	32.0	?
1620-Ep (E4)	+	—	12/29/67	32.3	?
1624-E2	1	—	5/13/68	33.5	?
1718-E1	—	4	10/29/68	26.8	4.04
1828-E1	—	8	3/11/69	19.8	4.22
1922-E1	—	1	8/14/67	24.7	3.75
2024-E1	—	1	3/27/68	21.4	3.29
2024-E5	—	1	3/27/68	23.2	?
2122-E2	—	3	1/7/69	26.8	?
2122-C (E3)	—	+	1/7/69	23.8	?
2126-E4	—	9	2/7/66	20.4	2.73
2224-E1	—	6	3/27/69	20.4	2.72

Peloscolex gabriellae var. *apectinata* Brinkhurst, 1965, page 135.
Peloscolex gabriellae apectinata. Brinkhurst and Cook, 1966, page 17.
Peloscolex apectinatus. Brinkhurst and Simmons, 1968, page 187.

DISTRIBUTION: see Table I (P.5) for Cape Cod Bay; Halifax Harbor, Nova Scotia; San Francisco Bay, California.

Peloscolex nerthoides Brinkhurst, 1965

Peloscolex gabriellae var. *nerthoides* Brinkhurst, 1965, page 134.
Peloscolex gabriellae nerthoides. Brinkhurst and Cook, 1966, page 17.
Peloscolex nerthoides. Brinkhurst and Simmons, 1968, page 187.

DISTRIBUTION: Station 0714-E4 (about 400 individuals) in Cape Cod Bay; San Francisco Bay, California.

Peloscolex intermedius Cook, 1969

Peloscolex intermedius Cook, 1969, page 11, Figure 2.

DISTRIBUTION: see Table II (P.6) and Figure 3 for Cape Cod Bay; continental shelf, near the continental slope, south of Martha's Vineyard, Massachusetts (Cook, 1970).

Limnodriloides medioporus Cook, 1969

Limnodriloides medioporus Cook, 1969, page 21, Figure 7.

DISTRIBUTION: see Table II (L.7) and Figure 3 for Cape Cod Bay; continental shelf, south of Martha's Vineyard, Massachusetts (Cook, 1970).

ECOLOGICAL SECTION

Sediment analysis

Mechanical analysis of the sediments in Cape Cod Bay was undertaken by Mr. A. Michael (Systematics-Ecology Program, Marine Biological Laboratory) using graded sieves for the coarse fraction (-1 to $+4\,\phi$) and pipette analysis for the fine fraction; the data, expressed in terms of ϕ units ($\log_2$ particle size in mm) were analyzed by the Woods Hole Oceanographic Institution's Sigma 7 computer. The results reveal that, except for the very coarse sediments (-1 to $+1\,\phi$), there is a general decrease in particle size with increasing depth (Fig. 4).

In the following discussion the median particle size (*i.e.*, median ϕ) is used throughout as a single, convenient parameter expressing the sediment type. Coarse sands (-1 to $2\,\phi$) are restricted to the eastern part of the bay and, in smaller areas, the mouth of Barnstable Harbor and the southwestern edge of the bay. Fine sands (2 to $4\,\phi$) are localized in the southern half, while silts and clays predominate in the central and northern parts of Cape Cod Bay (Fig. 2).

Distribution of the Tubificidae

The occurrence of the ten tubificid species was correlated with depth and the median particle size of the substrate (Table I and II, Fig. 4, and under "Distribution" of various species). These results, together with the mapped distribution of three of the dominant species (Fig. 3), show that the Tubificidae are divisible into two distinct, non-overlapping communities; (1) a fine-sand and silt community (3 to $7\,\phi$) consisting of *Peloscolex intermedius* and *Limnodriloides medio-*

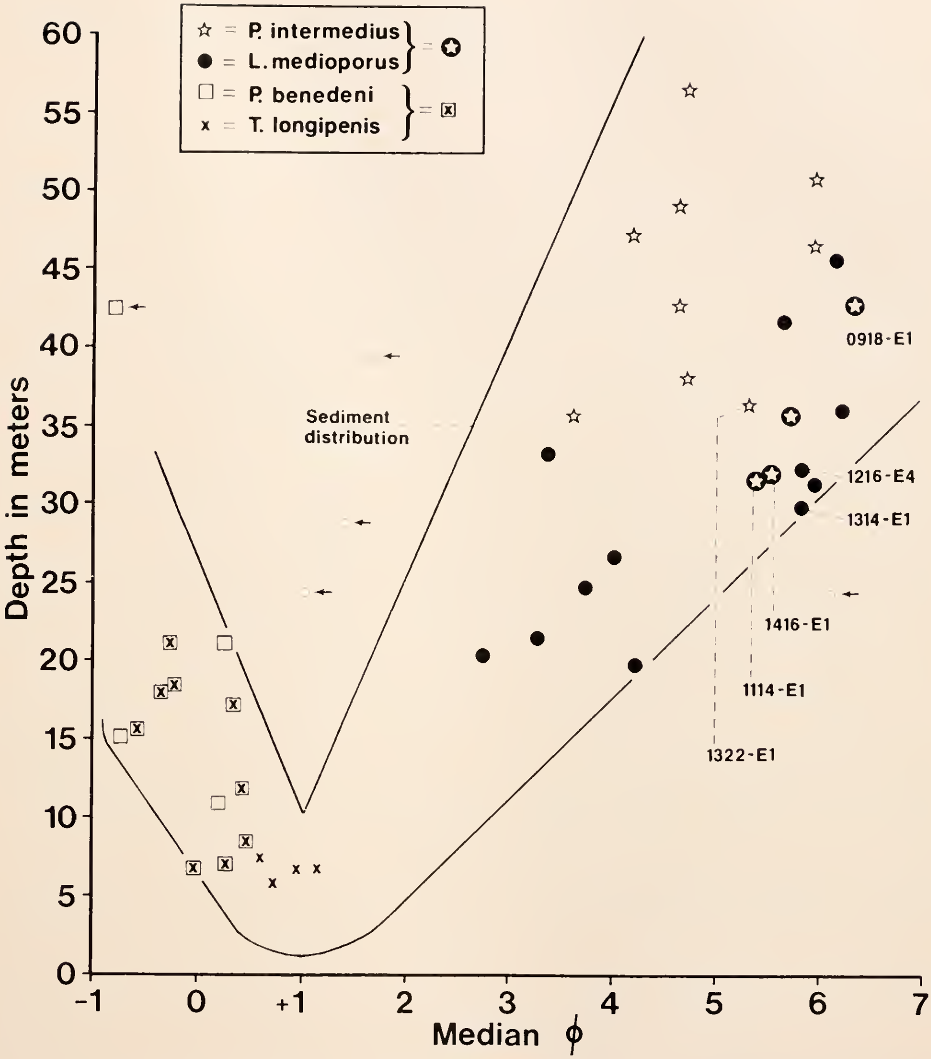

FIGURE 4. Distribution of the four dominant tubificids, *Peloscolex intermedius, P. benedeni, Limnodriloides medioporous* and *Tubifex longipenis,* in relation to the depth and median particle size of the substrate. The outer solid line indicates the outer limits of sediment types in relation to depth, with the exception of five stations (indicated by arrows) which lie outside the limits; a total of 136 stations were analysed for particle size.

porus; (2) a coarse-sand community (−1 to 2 ϕ consisting of *Phallodrilus obscurus, P. coeloprostatus, P. parviatriatus, Adelodrilus anisosetosus, Tubifex longipenis, Peloscolex benedeni, P. apectinatus* and *P. nerthoides.* Depth does not seem to be a limiting factor as the shallow limits of *L. medioporus* overlap with the deeper stations in which *P. benedeni* and *T. longipenis* occur (Fig. 4). The sands with a median ϕ of 1.2 to 2.7 are not inhabited by Tubificidae.

Whilst these two communities are distinctly separate in terms of particle size alone, the distributions of the species within them are more difficult to interpret and, therefore, will be considered separately.

(1) *Fine-sand and silt community*

P. intermedius and *L. medioporus* are the only two tubificids which occur in the finer sediments of Cape Cod Bay. As far as is known both species are unspecialzed infaunal deposit feeders. While apparently utilizing the same resources, the two species coexist in the central part of the bay (Fig. 2). Other such tubificid associations are well-known; *e.g.,* the fresh-water species *Limodrilus hoffmeisteri* and *Tubifex tubifex*. One mechanism for exploiting the same nutritional resource consists of a differential survival of various species of bacteria passing through the gut of different tubificid species (Brinkhurst and Chua, 1969), hence the results discussed below, based on sedimentary parameters, should be regarded as empirical rather than as an explanation of the mechanism of coexistence.

From the available data, the habitat of *P. intermedius* can be defined by depths in excess of 27 m and a substrate with median particle sizes ranging from 3.5 to 6.4 ϕ. *L. medioporus* occurs from 18 to 46 m in depth, and 2.7 to 6.4 median ϕ. The distribution of the two species can be summarized as follows: (1) *P. intermedius* and *L. medioporus* may occur either separately, or, at stations with fine substrates (5.3 to 6.4 ϕ), within the same 0.1 m^2 (Table II; Fig. 3); (2) both species reach their maximum abundance (up to about 2000 per m^2 for *L. medioporus*) in the central part of the bay (Table II) within a narrow range of substrate types, both in terms of median particle size (5.3 to 6.0 ϕ—Table II and Fig. 4), and total substrate composition (sand = 3 to 32%, silt = 50 to 72%, clay = 13 to 30%—Table II and Fig. 5); (3) the zone of maximum abundance physically overlaps the zone of species coexistence but not necessarily within the same 0.1 m^2 (Table II and Fig. 3); (4) both in terms of median particle size (Fig. 4) and total sediment composition (Fig. 5), *P. intermedius* can exist over most of the range of *L. medioporus* though the latter seems to be more tolerant of coarser sediments; (5) *L. medioporus* is often physically separate from *P. intermedius* as it tends to inhabit shallower stations especially when it occurs in the coarser sediments (Fig. 4); (6) *L. medioporus* appears to be absent in intermediate substrates with the median ϕ between 4.3 to 5.3 which are inhabited by *P. intermedius* (Fig. 4); (7) possibly identical to number 6 but stated in different terms, *P. intermedius* can exist in intermediate sediments (defined by median ϕ) with a lower percentage of clay than *L. medioporus* (Fig. 5).

A working hypothesis on the dynamics of this situation can be deduced from these facts, thus: an optimum sediment type, consisting of small particles which probably constitute an abundant food resource, allows *L. medioporus* and *P. intermedius* to coexist in relatively large numbers (1, 2, and 3), *P. intermedius* competitively excludes *L. medioporus* from sediments with average particle sizes only slightly larger than the optimum (6 and 7), and either (a) *L. medioporus* is more successful in exploiting coarser sediments and shallower environments than is *P. intermedius*, thus excluding competitively the latter from such situations, or (b) *P. intermedius* cannot survive depths less than about 25 m (4 and 5). It is impossible, with the present data, to decide whether competitive exclusion, the depth

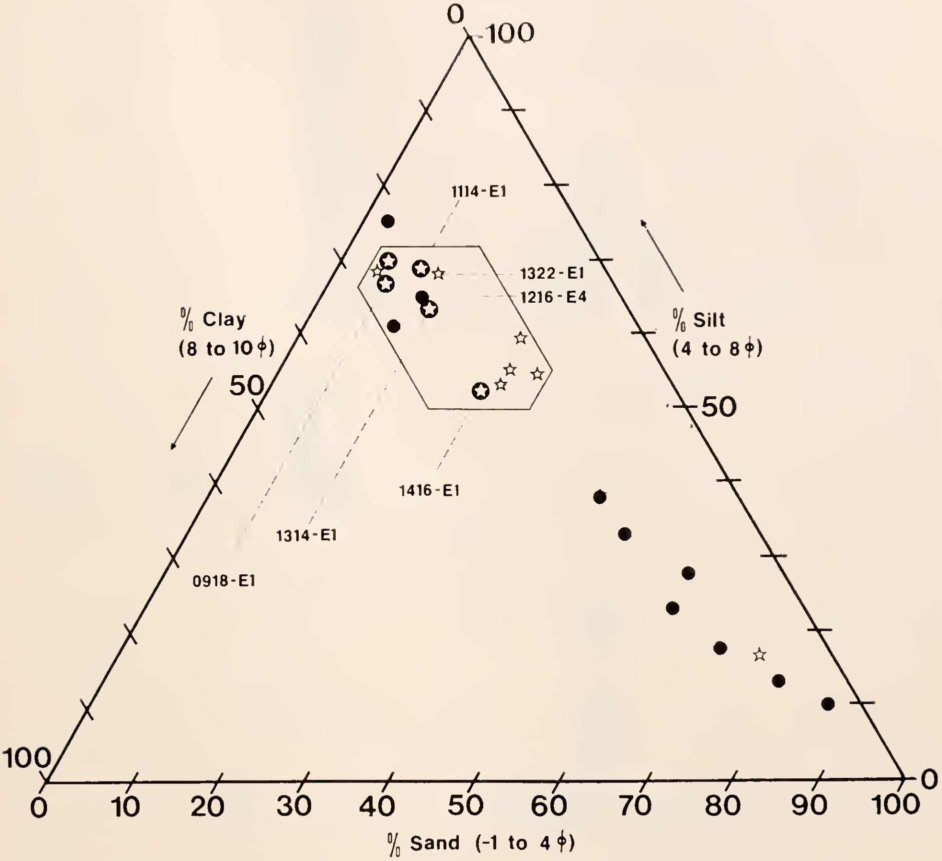

FIGURE 5. Distribution of *Peloscolex intermedius* and *Limnodriloides medioporus* in relation to the total sediment composition measured in percentages of sand, silt and clay. The symbols used for species identification are the same as in Figure 4.

tolerance of *P. intermedius*, or some other factor, is the deciding one in the distribution of this species and *L. medioporus* in the coarser sediments.

(2) *Coarse-sand community*

In the coarse sediments (from −0.80 to 1.83 median ϕ) *P. benedeni* is the dominant tubificid in both frequency and abundance (up to about 8000 per m^2) followed closely by *T. longipenis* (up to about 2300 per m^2) (Table I). The other six species which constitute the coarse-sand Tubificidae (*Adelodrilus anisosetosus, Phallodrilus obscurus, P. coeloprostatus, P. parviatriatus, Peloscolex apectinatus,* and *P. nerthoides*) occur sporadically (Table I and Systematic Section) but often in significant numbers. At any given station only one species may be present, or up to six can occur in association (*e.g.*, 1412-E4 where the total abundance of Tubificidae is about 10,000 per m^2; this is probably a low estimate because many,

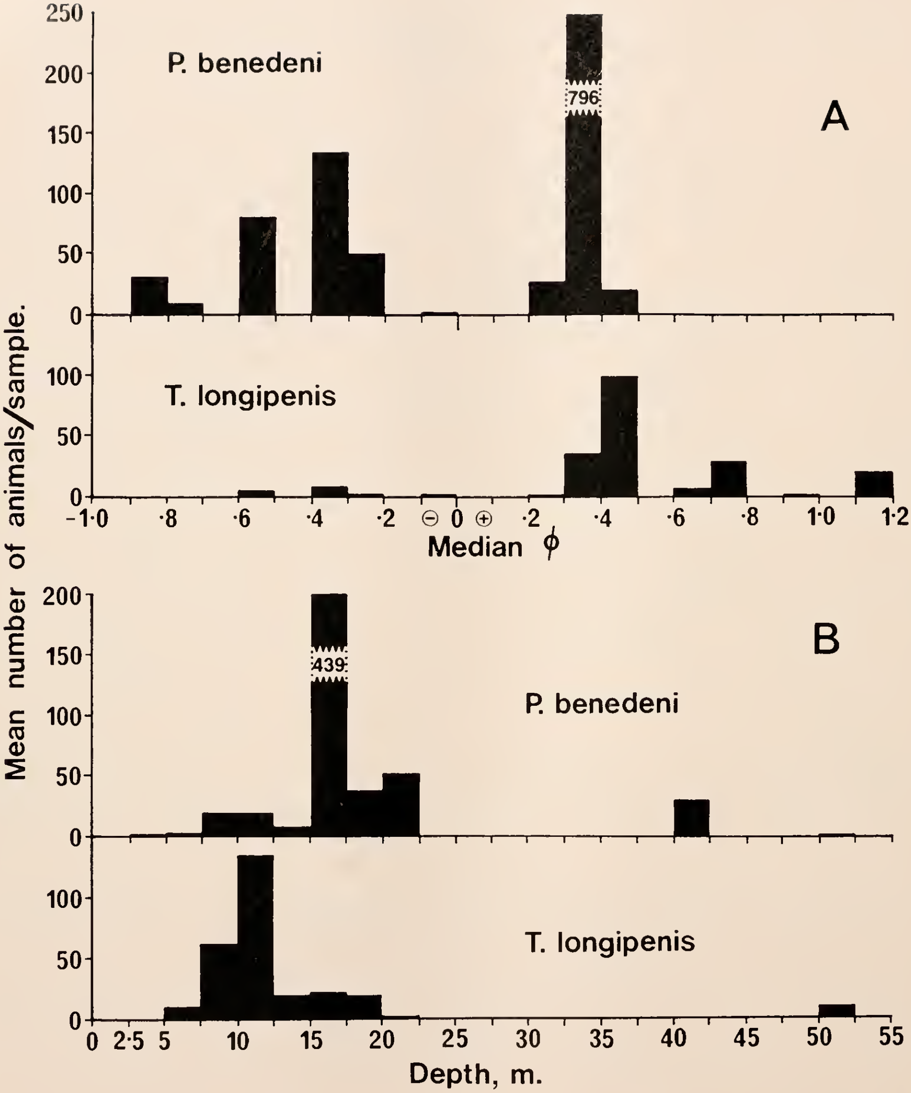

FIGURE 6. Histograms of the mean number of *Peloscolex benedeni* and *Tubifex longipenis* occurring in each quantitative sample in relation to two parameters: (a.) Median particle size (system of 0.1 ϕ classes used); (b.) Depth (2.5 m classes).

if not the majority, of the smaller species may not have been retained by the 1.0 mm screen).

In this section the two dominant species will be considered together, and brief notes on the other six, for which data are rather sparse, discussed separately. This arrangement is also convenient and fortunate from the biological viewpoint because *P. benedeni* and *T. longipenis* are probably both free-burrowing forms, being the counterparts of *P. intermedius* and *L. medioporus* in the fine-sands and silts, while all, or some, of the other six species, because of their small size, are likely to be members of the true interstitial fauna.

P. benedeni and *T. longipenis*

The distribution patterns of *P. benedeni* and *T. longipenis,* and their interpretation, bear some resemblance to those of *P. intermedius* and *L. medioporus*. The habitat of *P. benedeni* in Cape Cod Bay is characterized by depths between 4.3 to 51.2 m, and a substrate with the median particle sizes between -0.80 to $0.48\,\phi$; *T. longipenis* is found in depths between 5.8 to 51.2 and in sediments of -0.57 to 1.12 median ϕ. The distribution of the two species can be summarized as follows: (1) *P. benedeni* and *T. longipenis* occur either separately, or within the same 0.1 m^2 (Table I); (2) in terms of the median particle size of the sediment, *P. benedeni* is most abundant around 0.3 to $0.4\,\phi$, and *T. longipenis* between 0.4 to $0.5\,\phi$ (Fig. 6a); (3) *P. benedeni* occurs in high numbers in sediments coarser than $0.3\,\phi$ (especially between -0.6 to $-0.2\,\phi$) while *T. longipenis* has secondary peaks of abundance in sediments finer than $0.5\,\phi$ (between 0.6 to $1.2\,\phi$) (Fig. 6a); (4) in terms of depth, *P. benedeni* reaches its maximum abundance between 15 to 22.5 m, while 81% of the *T. longipenis* population exists between 2.5 to 15 m (Fig. 6b); (5) *T. longipenis* tends to occur alone in the finer substrates at the shallower stations (Fig. 4); (6) in terms of total sediment composition, all stations at which either, or both, species occur, lie in the category of sand = 97 to 100%, silt = 0 to 2% and clay = 0 to 1.8%.

These differences between the distributions of *P. benedeni* and *T. longipenis* may be interpreted as follows: the two species may coexist over a wide range of coarse sediments but *T. longipenis* seems more able to exploit those with finer particles which tend to occur at shallower stations (Fig. 4), possibly excluding *P. benedeni* from them. Similarly they can exist together over a wide depth range; the deep limits in Cape Cod Bay are probably set by the distribution of suitable sediments (Fig. 4); the shallow depth limits *per se*, contrary to the implication of Figure 6b, are not thought to be controlling factors because of the high correlation between depth and sediment composition (Fig. 4), and the fact that *P. benedeni* is known to extend well up into the littoral zone in other localities (Moore, 1905; Lasserre, 1967).

Remaining coarse-sand Tubificidae

Little can be said concerning the remaining six species on the limited data available; all occurred within the depth and median particle size ranges of the two dominant species, except *Phallodrilus coeloprostatus* (see below and Table I). Geographically the majority of these species were concentrated in a small ovoid area of Cape Cod Bay whose major axis runs from 1412-E2 in the northeast, to 1816-E1 in the southwest (Fig. 3). The upper two-thirds of this area corresponds to the concentration of very coarse sediments (-1 to $0\,\phi$; Fig. 2).

Phallodrilus coeloprostatus: This species, next in order of numerical importance after *P. benedeni* and *T. longipenis*, occurred at fourteen stations, at eleven of which it was associated with *P. benedeni*. It extends into slightly finer sediments ($1.37\,\phi$) than the two dominant species and, like these, its lower depth limit in the bay (51.2 m) is probably a function of substrate availability. Geographically *P. coeloprostatus* occurred within or near the ovoid area of maximum species concentration (hereafter called area "0"), a small near-shore area at the northwestern tip of Cape Cod (0616), and an isolated area near the western shore (1930).

Phallodrilus parviatriatus: P. parviatriatus occurred at only four stations where both *P. benedeni* and *T. longipenis* were also found and which all lie within area "0."

Phallodrilus obscurus: This species was found at only two stations located close to the western shore (6.7 and 8.5 m depth). It may be significant that both stations have relatively fine sediments (0.81 and 1.12 median ϕ) and that the *P. benedeni* population appears to be absent from this part of the bay.

Adelodrilus anisosetosus: Five out of the total of six stations at which this species occurred lie within area "0"; the isolated station on the western shore, like the other five, lie within the range, median $\phi = -0.28$ to 0.54, depth $= 10.4$ to 21.1 m.

Peloscolex apectinatus: The species was represented at six stations in area "0," at one station near the bay's northeastern edge, and at three stations along the western shore. All of these stations are shallower than 22 m and have median particle sizes finer than $-0.3\ \phi$.

Pelescolex nerthoides: This was found at only one station (0714-E4), but in considerable numbers (4000 + per m^2), at a depth of 42.4 m and in a very coarse sediment (-0.8 median ϕ).

Discussion

As a basis for discussion the major conclusions of the ecological section may be summarized as follows: within Cape Cod Bay two different communities of Tubificidae occur; species distribution, between and possibly within communities, is related to the median particle size of the sediments; (1) *P. intermedius* and *L. medioporus* constitute a free-burrowing, fine-sand and silt community; (2) *P. benedeni* and *T. longipenis* play the dominant role in a coarse-sand community which may be subdivided into (a) a free-burrowing component composed of the two dominant species, and (b) an interstitial component composed of six species characterized by their small size.

Marine Oligochaeta are not well-known and factors influencing their distribution have been little studied. Lasserre (1967) in an analysis of some littoral Enchytraeidae and Tubificidae concluded that their distribution was correlated with the median particle size of the substrate and the percentage of sediment under 80 μ ($3.64\ \phi$) in diameter. Lasserre (1967) found *P. benedeni* in sediments with median particle sizes ranging from -1.0 to $1.74\ \phi$ (compared to -0.8 to $0.48\ \phi$ in the present study), at densities up to 1,000,000 per m^2 in the finer sediments with 7 to 10% of the particles smaller than $3.64\ \phi$: densities declined in coarser substrates and as the percentage of particles smaller than $3.64\ \phi$ declined. In the present study, high densities of *P. benedeni* also tended to occur in the finer sediments: the fact that *P. benedeni* was not found in sediments finer than $0.48\ \phi$ (compared to $1.74\ \phi$ in Lasserre's work), supports the hypothesis that the species may be competitively excluded by *T. longipenis* in the finer sediments.

Other authors mentioning sediment types in relation to marine or littoral Oligochaeta include Bülow (1957) who demonstrated that zones of differing substrata supported different oligochaete faunas, Brinkhurst and Kennedy (1962) who found that the relative abundance of three tubificids was controlled in part by the substrate, and Brinkhurst (1964a) who noted that *Tubifex costatus* (Claparède, 1863) inhabited a wide variety of sediment types.

The fresh-water Tubificidae, however, have been better studied, especially in recent years: species distribution and/or abundance is controlled, according to Ravera (1951) by the organic content of the sediment, Henson (1963) stated that factors other than inorganic parameters are operative, and Brinkhurst (1962; 1964b) concluded that, while the depth and the nature of the substrate may be determinants of some species distributions, biotic factors (interspecific competition and predation) are probably the most important. Valle (1927), Rzoska (1936), Szczepanski (1953), Della Croce (1955), Korn (1963) and Wachs (1967; 1968) have all shown a positive correlation between species distributions and inorganic sediment characteristics in fresh-water.

Thus, many of the studies on both fresh-water and marine Tubificidae have concluded that a correlation exists between the substrate type and the distribution and/or abundance of species. This work reports similar results, but Brinkhurst (1962) has pointed out that observed species distributions, however closely they may seem to be correlated with sediment types, may be a reflection of some other factor such as the distribution of predators. This is especially true in the case of the marine Oligochaeta, a group about which such basic information as, their tolerance to physical conditions, their specialized food requirements, and the identity of their predators, is still unavailable. However, despite these reservations, the results discussed here, whilst failing to elucidate definitively the autecology of the Tubificidae in Cape Cod Bay, do have an empirical validity which, for a rather obscure but ecologically significant group of marine animals, may be sufficient justification for their presentation.

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Summary

(1) Ten species of Tubificidae are recorded from Cape Cod Bay, as follows: *Adelodrilus anisosetosus, Limnodriloides medioporous, Peloscolex apectinatus, P. benedeni, P. intermedius, P. nerthoides, Phallodrilus coeloprostatus, P. obscurus, P. parviatriatus* and *Tubifex longipenis.*

(2) *Phallodrilus parviatriatus* nov. sp. is characterized by small erect atria and simple-pointed posterior setae.

(3) The original description of *Tubifex longipenis* Brinkhurst, 1965, is confirmed and expanded.

(4) On the basis of species distributions in relation to sediment types, two major tubificid communities are recognized.

(5) *P. intermedius* and *L. medioporus* constitute a free-burrowing, fine-sand and silt community (median particle size of the substrate between 3 to 7 ϕ).

(6) *P. benedeni* and *T. longipenis* are the free-burrowing components of a coarse-sand community (-1 to 2 median ϕ) which also contains *A. anisosetosus, Peloscolex apectinatus, P. nerthoides, Phallodrilus coeloprostatus, P. obscurus* and *P. parviatriatus;* some, or all, of the last six species are probably interstitial.

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INTRACELLULAR DIGESTION OF SYMBIONTIC ZOOXANTHELLAE BY HOST AMOEBOCYTES IN GIANT CLAMS (BIVALVIA: TRIDACNIDAE), WITH A NOTE ON THE NUTRITIONAL ROLE OF THE HYPERTROPHIED SIPHONAL EPIDERMIS

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Zooxanthellae are highly specialized dinoflagellates which live mostly as endosymbionts within a wide phyletic range of marine invertebrate hosts (Buchner, 1965; McLaughlin and Zahl, 1966). The symbiosis between zooxanthellae and host animal is regarded by most workers as mutualistic; that is, a relationship which is beneficial to both host and symbiont. In exchange for protection, carbon dioxide, and nutrient salts provided by the host tissues, the symbiont releases small amounts of oxygen and an appreciable quantity of photosynthetic metabolites (principally glycerol) which are utilized by the host (Smith, Muscatine and Lewis, 1969; Goreau, Goreau and Yonge, 1965; Yonge, 1944).

Within the family Tridacnidae (see Fig. 1 for an example of this group), this relationship is further elaborated. From histological evidence, Yonge (1936 and 1953) has concluded that zooxanthellae are conveyed within amoebocytes from the hypertrophied siphonal haemal spaces of *Tridacna* where they are "farmed" by the clam, to the interdiverticular spaces of the clam's digestive gland. Further, these engulfed zooxanthellae are intracellularly digested by the amoebocytes both *en route* via blood vessels from the mantle and within the interdiverticular spaces of the digestive gland.

Mansour's accounts (1945, 1946 and 1949) are in disagreement with Yonge's findings. Zooxanthellae were never found within the amoebocytes of tridacnid clams by Mansour. Moreover, it was solely in young or juvenile tridacnids that zooxanthellae were observed by him within tissues of the visceral mass. In no case were algal cells present in blood vessels; *ergo*, concludes Mansour, zooxanthellae could not, as claimed by Yonge (1936 and 1953), be transported from the siphons to the visceral mass via the circulatory system.

The utilization of symbiontic algae as a holozoic food source by their invertebrate hosts has been recently reviewed (Smith, Muscatine and Lewis, 1969), and with regard to the acquisition of insoluble carbohydrate by the host as a result of degeneration and digestion of whole algal cells or their parts, it was stated that the critical evidence is still lacking. The purpose of this report is, hopefully, to settle this question, at least with respect to tridacnid clams, by means of the more precise techniques of electron microscopy and electron microscopical histochemistry. In addition, the possible nutritive role of the epidermis covering the hypertrophied siphons will be discussed.

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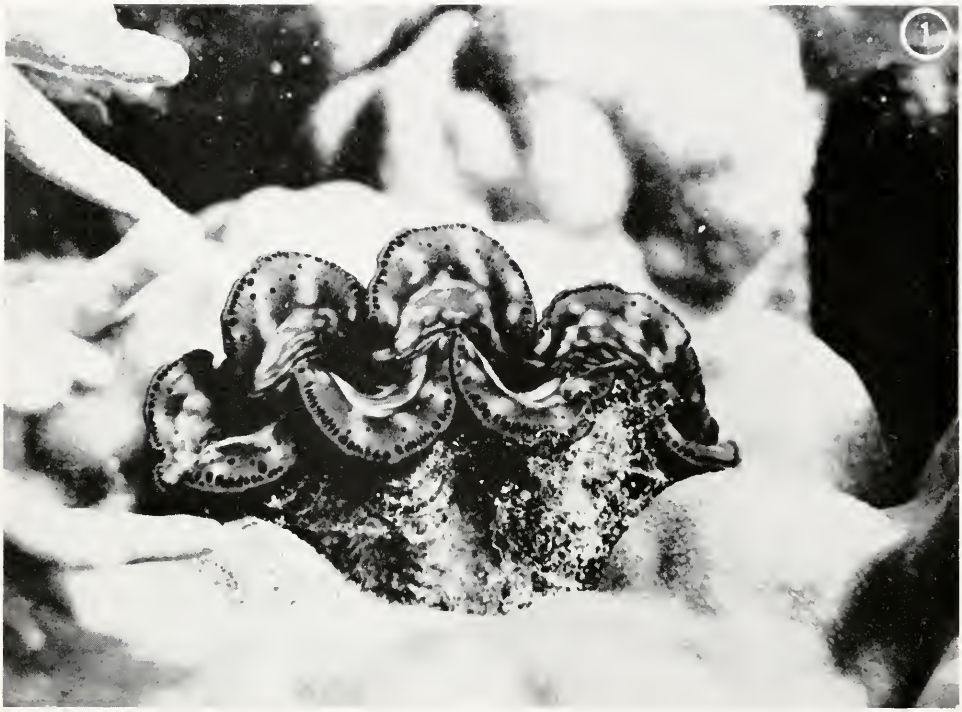


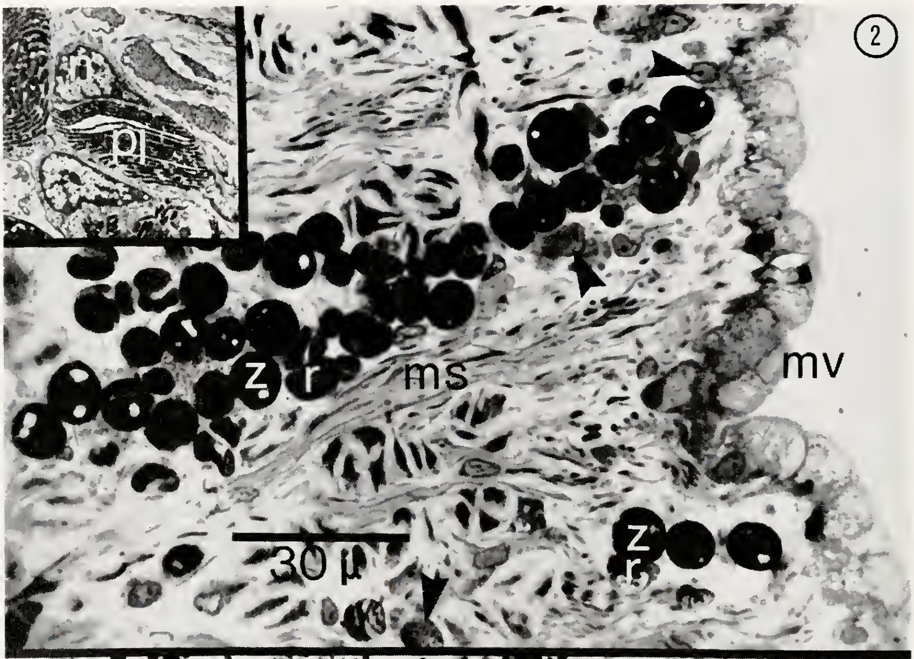
FIGURE 1. In water photograph of one of the more diminutive species of tridacnid clams, *Tridacna maxima* taken adjacent to Chinimi Islet, Eniwetok Atoll, Marshall Islands. The clam lies byssally attached to coral incrusted limestone with its hypertrophied siphons (the whole of the fleshy portion in the figure) fully exposed to the sun's rays.

MATERIALS AND METHODS

Adult specimens of the tridacnid clams *Hippopus hippopus, Tridacna gigas, T. maxima,* and *T. squamosa* (Rosewater, 1965), were collected in shallow coral reef waters at Eniwetok Atoll, Marshall Islands, by free diving or using S. C. U. B. A. Blood samples from the circulatory systems were syringe extracted from the clams' hearts and immediately centrifuged to precipitate a firm easily handled pellet of amoebocytes. One millimeter square cubes of clam digestive gland and the mantle edge inner folds (*Hippopus* excluded) were cut from tridacnids and, along with the centrifuged amoebocyte fractions, were treated in one or both of the following ways:

Method I—light and electron microscopy

1. Fixed at 0° C for $1\frac{1}{2}$ hours in 2% osmium tetroxide buffered with Dorey's solution B (1965). For better penetration and osmotic compatibity of fixative with tissues, 3.5% potassium chloride was substituted for the sucrose originally prescribed in the Dorey formula.
2. Dehydrated and embedded with Epon 812 (Luft, 1961).



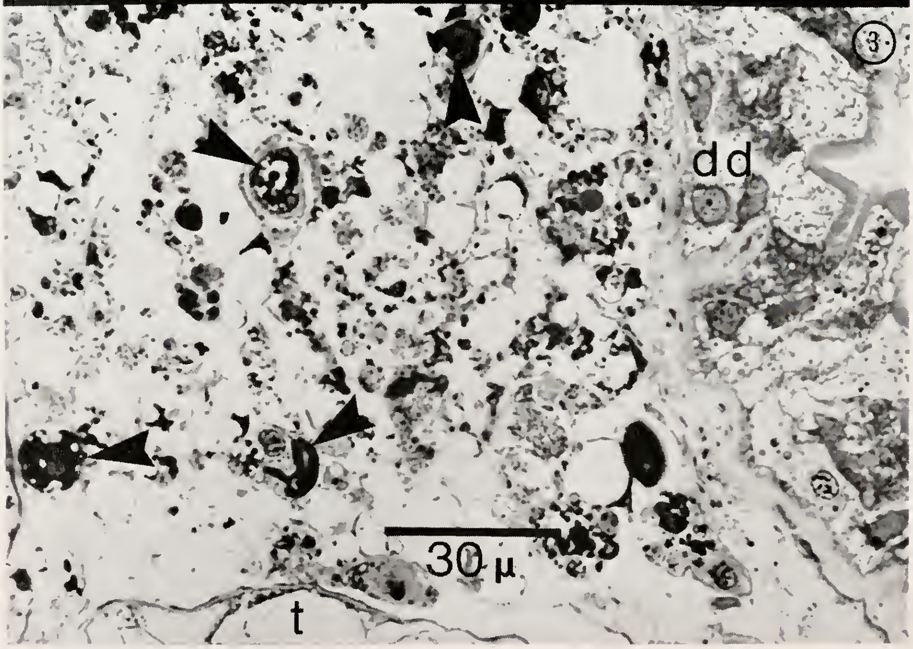


FIGURE 2. Siphonal tissue of *Tridacna maxima* hosting symbiotic zooxanthellae within haemal channels. Insert top left: Fine structure of iridophore from siphonal tissues. Abbreviations used are: ms, muscle strands; mv, microvillous surface of vacuolated epidermal cells; n, nucleus; pl, plates; r, iridophore; z, zooxanthellae; point, amoebocyte.

3. Gray to silver sections were cut and stained with lead and uranium for electron microscopy. For conventional bright field light microscopy, one micron thick sections were cut, affixed to microscope slides and stained with Richardson's stain (Richardson, Jarett, and Finke, 1960).

Method II—histochemical test for phosphomonoesterase II (acid phosphotase)

1. Fixed at 0° C for 2 hours in 6% glutaraldehyde buffered with 0.2 M cacodylate at pH 7.4. The 6% glutaraldehyde solution was made from a 25% prebuffered (3% calcium carbonate) stock solution of "Fisher" biological grade glutaraldehyde.
2. Washed at 0° C overnight in 0.2 M cacodylate buffer (pH 7.4.).
3. Incubated in fresh filtered Gomori's medium (1950) at 37° C for 1½ hours. The β-glycerophosphate constituent of the Gomori medium should contain no more than 0.1% L-α-isomer impurity for best results.
4. Washed as follows: acetate buffer, pH 5.0, 15 minutes; 2% acetic acid, 3 minutes; acetic acid, 3 minutes; acetate buffer, pH 7.2, 10 minutes.
5. Postosmicated, dehydrated, and embedded as described in Method I, above.
6. For electron microscopy, silver-gold thin sections were cut and left unstained.
7. The control incubation medium consisted of the Gomori medium with 0.42% sodium fluoride added as an enzyme inhibitor.

I have drawn liberally upon the papers of Kevin, Hall, McLaughlin and Zahl (1969) and Taylor (1969a) for identification of fine structure in zooxanthellae.

Results

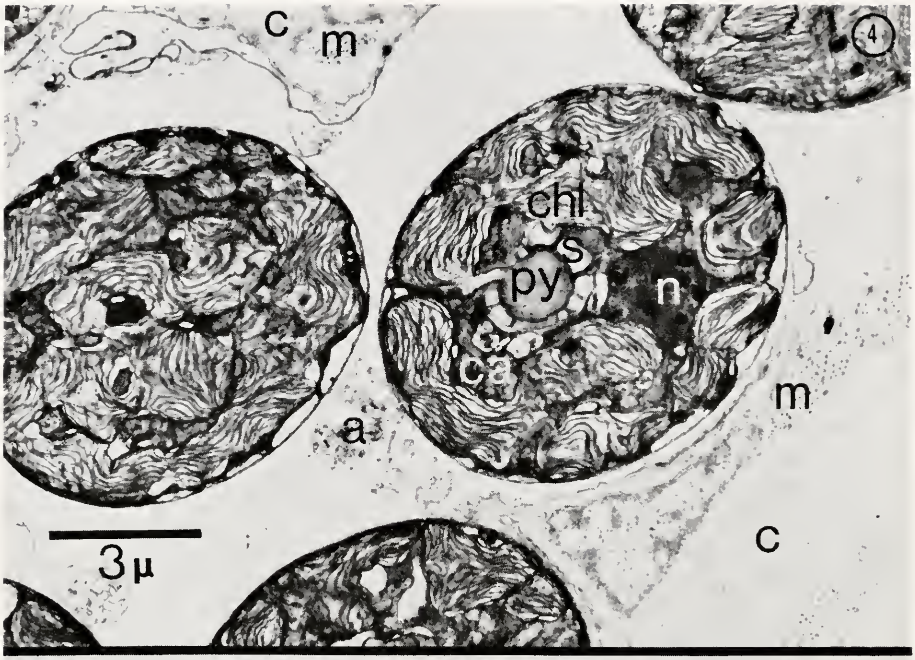
The hypertrophied siphonal tissues of *Tridacna* are comprised chiefly of a muscle and connective tissue meshwork covered by a narrow undulant veneer of highly vacuolated microvillous epidermal cells (Fig. 2). Symbiontic zooxanthellae live closely appressed within host haemal channels, which are arranged approximately perpendicular to the siphon's exposed surface. Scattered amongst the zooxanthellae (z) are various amoebocytes (a) and iridophores (r). Peculiar to the cytoplasm of iridophores are stacks of parallel electron dense plates (pl) (Fig. 2) to which Kawaguti (1966) attributes the siphon's iridescence.

Light microscope studies revealed that zooxanthellae of the interdiverticular spaces of the digestive gland (Fig. 3) and circulatory system are dissimilar in their gross histology from those found in the haemal channels of the siphon's. Algal cells from the siphons are uniformly ovoid in shape and possess a homogeneous cytoplasm rift with chloroplasts. In contrast to this, zooxanthellae seen in the circulatory system and seen in the interdiverticular spaces of the digestive gland may be observed in various conditions: ranging from free, inclusion-bearing orbs to barely recognizable deformed bodies which are undergoing the later stages of intracellular digestion by amoebocytes.

Ultrastructure of zooxanthellae living within tridacnid siphonal tissues

Electron photomicrographs demonstrated that siphonal zooxanthellae are adjacent to but never completely enclosed by amoebocytes (a), muscle strands (m),

FIGURE 3. Amoebocyte packed interdiverticular space of digestive gland of *Tridacna squamosa*. Abbreviations used are: dd, duct of digestive diverticula; t, tubule of digestive diverticula; points, digesting zooxanthellae contained within amoebocyte digestive vacuoles.



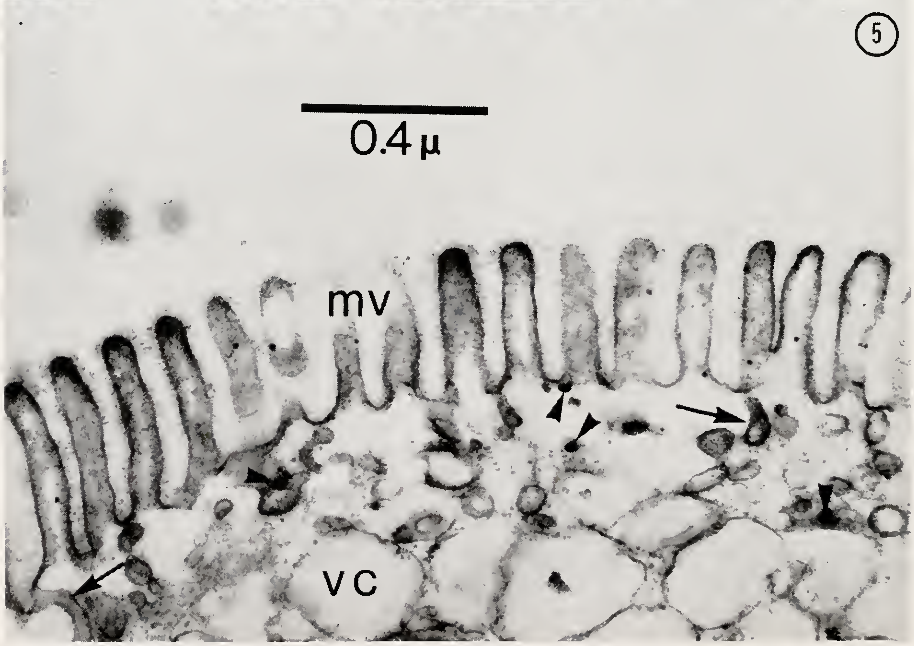


FIGURE 4. Ultrastructure of zooxanthellae within siphonal haemal space of *Tridacna maxima.* Note that zooxanthellae are adjacent, but not engulfed by amoebocytes. Abbreviations used are: a, portion of amoebocyte; c, connective tissue; ca, calcium oxalate crystals; chl, chloroplast; m, muscle strand; n, nucleus; py, pyrenoid body; s, starch cap.

connective tissue (c) (Fig. 4), or iridophores. Three unit membranes, comprising the zooxanthella's periplast, surround the chloroplast-dominated cytoplasm of the alga. The chloroplasts (chl) are individually joined by a short neck to a starch-capped pyrenoid body (py). Other cytoplasmic organelles which may be included are: a stellate-shaped nucleus (n), invested with numerous spring-shaped chromosomes; a small waste vacuole containing calcium oxalate crystals (ca); mitochondria, frequently multilobed; a small electron dense accumulation body; and, often, a four to six dictyosome golgi complex. I observed no obviously degenerate or senile zooxanthellae within siphonal tissues.

Uptake by microvilli covering the epidermis of tridacnid siphons

Microvillous epidermal cells of the hypertrophied siphons of *Tridacna* pinocytose phenomenal quantities of both fluid and particulate substances from the seawater which bathes the siphon's surface (Fig. 5). I use the term phenomenal advisedly because by my rough but conservative estimates, a 20 centimeter long specimen of *Tridacna maxima* possesses a total exposed siphonal surface area of over 1000 square centimeters. I could see evidence of fusion of pinocytotic vesicles with the epidermal cell's omnipresent clear cytoplasmic vacuoles, but the ultimate disposition of this endocysted material was not determined.

Intracellular digestion of zooxanthellae by amoebocytes lying within the interdiverticular spaces of the tridacnid digestive gland

The interdiverticular spaces of the tridacnid digestive gland are solidly packed with amoebocytes, many of which contain one or two zooxanthellae in various stages of intracellular digestion (Yonge, 1936). Free, or non-digesting, zooxanthellae are not commonplace in either the digestive gland or the circulatory system, but they do infrequently occur. However, whether free or engulfed, provided that, in the latter instance, intracellular digestion has not progressed beyond the early stages, nearly all of these zooxanthellae share recognizable cytological characteristics not common to their counterparts in the mantle edge. For example, the outer covering, or periplast, is greatly thickened due to the addition of a wide band of semi-opaque material sandwiched between the three original bounding membranes plus one or two more coated onto the periphery (Fig. 6). Another obvious periplast feature of many of the zooxanthellae undergoing digestion is the presence of vacuole-like foldings on the inside boundary of the opaque layer (Fig. 8). Presumably these structures are derivatives of the original periplast membranes. The chloroplasts (chl), now more electron dense than those seen in the zooxanthellae of the mantle, no longer predominate in the cytoplasm, but have been, in part, supplanted by much enlarged accumulation (ab) and calcium oxalate (ca) crystal bodies (Figs. 6 and 7). The pyrenoid body is still in evidence in some sections, but the starch cap has been assimilated by the zooxanthella. In many cases, lipid storage bodies are found throughout the cytoplasm.

Intracellular digestion of a zooxanthella by an amoebocyte is evidently initiated

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FIGURE 5. Pinocytosis of fluid and particulate matter by siphonal microvillous epidermal cells in *Tridacna maxima*. Abbreviations used are: mv, microvillous surface of vacuolated epidermal cells; vc, clear vacuoles; arrows, fluid filled pinocytotic channels; points, pinocytosis of particulate material.

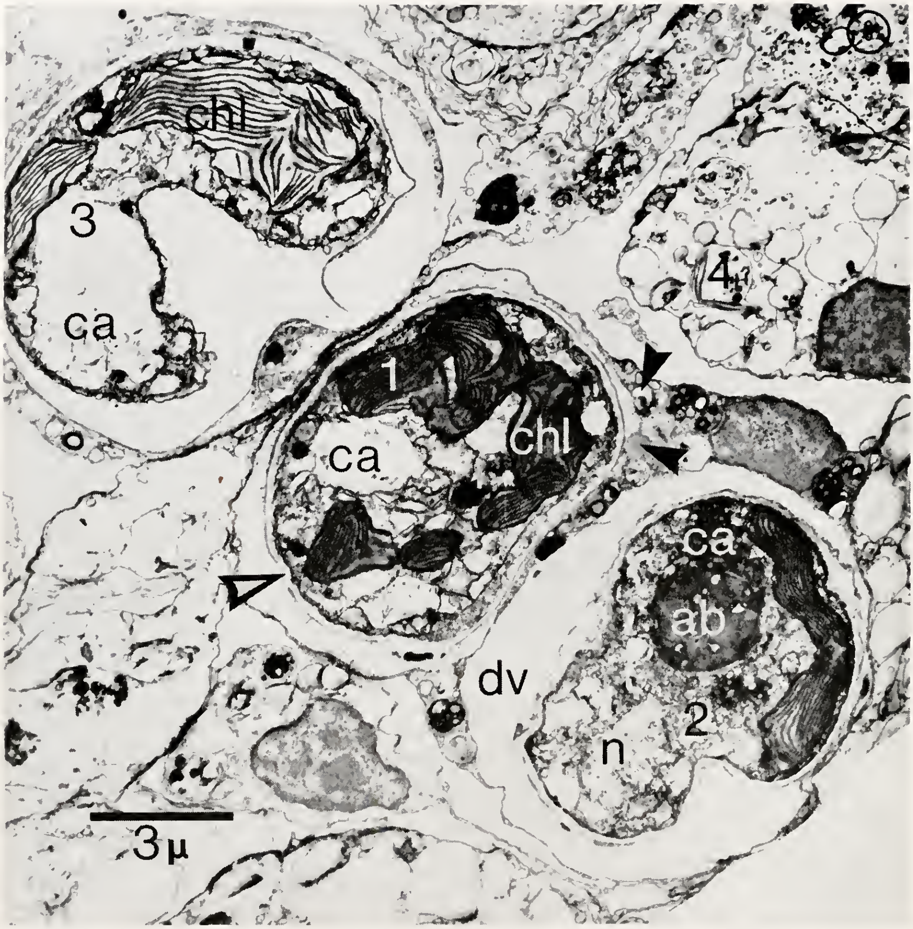


FIGURE 6. Enumerated successive stages of intracellular digestion of senescent zooxanthellae by amoebocytes lying within the interdiverticular spaces of the digestive gland of *Tridacna gigas.* (1), amoebocyte lysosomes burst their contents into vacuole containing the algal cell; (2), zooxanthella's periplast breaks down and cell outline becomes noticeably crenated; (3), chloroplast lamellae spread apart giving an overall bleached appearance to the zooxanthella; (4), digestive vacuole becomes reduced in size due to seepage of fluid nutrients to the amoebocyte's cytoplasm. Abbreviations used are: ab, accumulation body; ca, calcium oxalate crystals; chl, chloroplast; dv, amoebocyte digestive vacuole; n, nucleus; black points, amoebocyte lysosomes bursting contents into digestive vacuole; white on black point, amorphous layer of thick periplast.

when the amoebocyte's lysosomes burst their contents into the vacuole containing the algal cell; this vacuole is now termed the digestive vacuole (Fig. 6). Lysosome involvement in the digestive process was verified according to the terms of de Duve (1963) on the basis of both morphology and a positive histochemical test for the presence of phosphomonoesterase II (Fig. 7). In the second stage of intracellular digestion, the zooxanthella's periplast begins to break down and the algal cell has become noticeably crenated. Chloroplasts remain quite electron dense, but the general cytoplasmic detail has become muddied. The membranes enclosing the calcium oxalate crystal bodies have begun to part, appearing to release the vacuole contents into the cytoplasm (Figs. 6 and 8). An abrupt alteration takes place in the electron density of and in the degree of apposition of the chloroplast lamellae in the third stage of the digesting zooxanthella. Chloroplast lamellae now possess a bleached appearance and are widely separated. As digestion progresses, the amoebocyte's digestive vacuole(s) (dv) contents become much reduced in size due, presumably, to the seepage of the fluid nutrient matter from the digestive vacuole to the cytoplasm of the amoebocyte. The final visible remains are barely recognizable bits of chloroplast thylakoids, and these are soon reduced to myelin figures (d) (Fig. 8) in the now diminutive digestive vacuole.

Light and electron microscope observations on sections cut of the amoebocyte fraction of centrifuged clam blood revealed numerous zooxanthellae, many of which were in early stages of digestion within amoebocyte digestive vacuoles. However, the relative numbers of zooxanthella to total blood cells from the circulatory system was noticeably more sparse than that found within the interdiverticular spaces of the digestive gland.

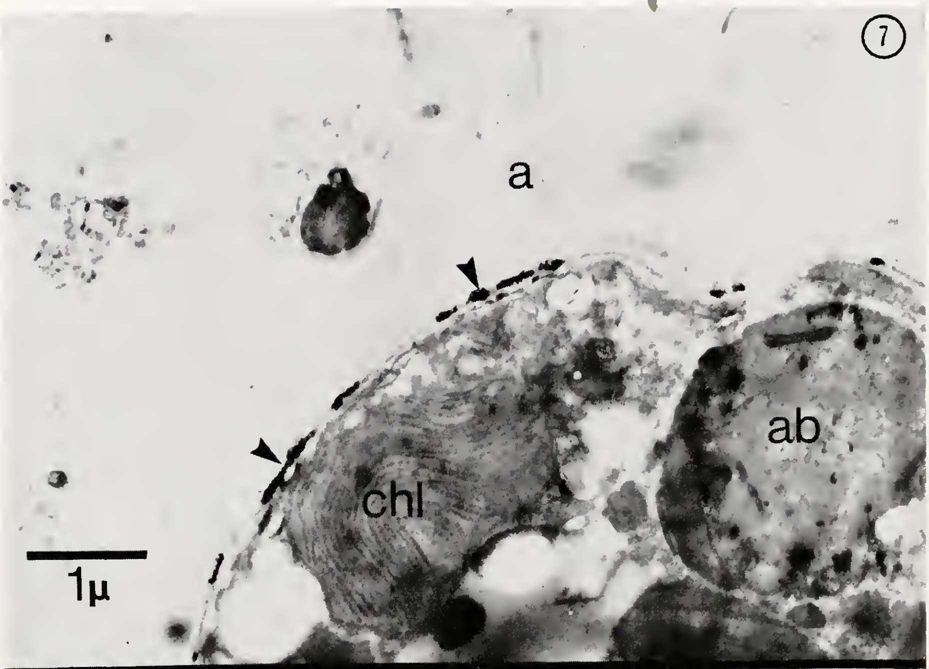
Reproduction of zooxanthellae (binary fission) was frequently observed in mantle edge tissue preparations, but in no instance did I find any suggestion of algal reproduction in tissues from either the digestive gland or the circulatory system.

Discussion

Senescence in a vegetative zooxanthella may be recognized by the following characteristics: a low chloroplast density; large accumulation and calcium oxalate crystal bodies; a thick periplast formed by five unit membranes; and a pyrenoid body (if present) with a reduced or missing starch cap (Freudenthal, 1962; Kevin, Hall, McLaughlin and Zahl, 1969; Taylor, 1968). These criteria coincide with my description and/or electron photomicrographs (Figs. 3, 6, 7 and 8) of zooxanthellae observed from both the circulatory system and the interdiverticular spaces of the digestive gland of tridacnid clams. Hence, it is apparent that amoebocytes in tridacnids phagocytose mostly old or degenerate zooxanthellae from the algal population of the mantle edge.

Yonge (1936) has suggested that tridacnid clams "farm" the zooxanthellae housed within their siphonal tissues as a holozoic food source. This being strictly so, why are tridacnid amoebocytes culling degenerate rather than young or mature zooxanthellae for subsequent intracellular digestion? From solely the nutritional standpoint, this arrangement doesn't make good sense. While mature zooxanthellae possess storage starch and a nutrient rich cytoplasm, senescent forms are usually comprised of a membraneous bag containing little more than calcium oxalate crystals plus the cell wastes (the accumulation bodies) of numerous generations of zooxanthellae (see Freudenthal, 1962; McLaughlin and Zahl, 1966 for details of reproductive biology of zooxanthellae). No doubt there is some nutritional value in a degenerate or senescent zooxanthellae, but surely nothing comparable to that found in a younger cell. Why then do amoebocytes appear to be selectively removing senescent zooxanthellae?

In Taylor's (1969b) study on regulation and maintenance of zooxanthellae in the coelenterate *Anemonia sulcata,* he notes that it is possible for degenerate algal



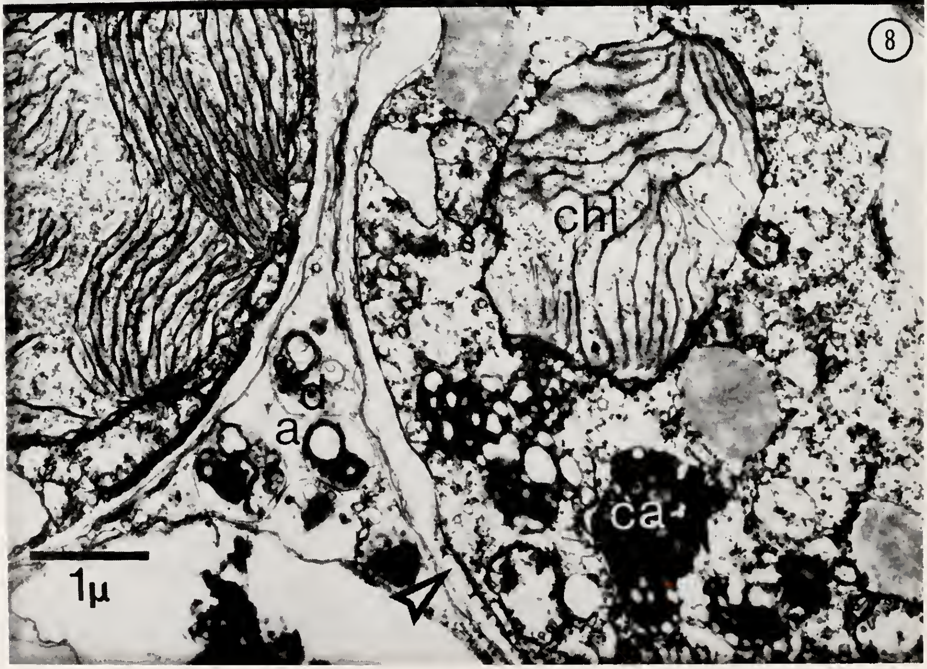


FIGURE 7. Unstained thin section of digestive gland tissue from *Hippopus hippopus* demonstrating sites of phosphomonoesterase II activity (points) during early stage of intracellular digestion of zooxanthella by amoebocyte lysosomes. Abbreviations used are: a, portion of amoebocyte; ab, accumulation body; chl, chloroplast.

cells to differ metabolically from healthy symbionts and, thus, represent a hazard to their host. *Anemonia* rids itself of degenerate zooxanthellae in the same manner as madreporarian corals (Yonge and Nicholls, 1931), by releasing them directly into the environment and thereby maintains a non-harmful host-symbiont balance. Tridacnid clams may confront a similar stress with a different solution—the use of amoebocytes to remove and dispose of zooxanthellae which no longer contribute to the clam's well being.

The criteria for selection or culling of zooxanthellae for sacrifice by tridacnid amoebocytes may well be due to some new expression of the alga's metabolism brought on by senility or degeneration. This new expression of metabolism (*i.e.*, change in a zooxanthella's outer membrane structure and/or a reduction in its release of photosynthetic metabolites to the host) might cause amoebocytes to recognize a senescent zooxanthella as neither host nor symbiont tissue and, thereupon, initiate phagocytosis to remove it from the clam's system.

Yonge (1936) argues in support of "farming" of zooxanthellae by tridacnids through calling attention to the immense size of tridacnid kidneys. He presumes that these organs, which are proportionally a magnitude in size larger than those found in other Lamellibranchs, store the indigestible wastes accumulating from countless instances of amoebocyte digestion of siphonal zooxanthellae. However, growth studies by Rosewater (1965) on *T. gigas* indicate that very large specimens of this clam are at least a quarter of a century old. It appears manifest that by adding this temporal consideration to Yonge's arguments, they become nearly untenable—it is more than a little difficult to visualize how the waste accumulation resultant from 25 years of "farming" could be housed within the kidney spaces of even a very large tridacnid clam.

Results of $^{14}CO_2$ labeling experiments on *Tridacna maxima* (Goreau, Goreau, and Yonge, 1965) suggest that the turnover rate of zooxanthellae in the interdiverticular spaces of the digestive gland is slow. Coupled with the results of the present paper, this suggests that while there may exist a true holozoic relationship between tridacnid clams and zooxanthellae, this relationship *cannot* be considered "farming," but rather the systematic removal *and utilization* of degenerate zooxanthellae from the mantle's algal population. In this respect, tridacnid clams appear to demonstrate a clear bioenergetic superiority over their coelenterate counterparts.

The evolution of hypertrophied siphons in tridacnids has allowed both greater exposure to solar radiation for proliferation of their algal populations (Yonge, 1936 and 1953) and the development of an extensive microvillous surface, which appears to possess a prodigious capacity for assimilation of both fluid and particulate matter from the surrounding seawater. The ultrastructure of the siphons of *Tridacna crocea* has been described by Kawaguti (1966), who mentions the presence of microvilli covering the siphonal epidermis in passing, but attaches no functional significance to these membraneous structures.

Uptake of nutrient material from seawater by microvillous epidermal cells has been clearly established in echinoderms, molluscs, and pogonophores (Fontaine

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FIGURE 8. Digestion of zooxanthellae by amoebocyte lying within the interdiverticular spaces of the digestive gland of *Tridacna maxima*. Abbreviations used are: a, portion of amoebocyte; ca, calcium oxalate crystals; chl, chloroplast; d, myelin figures in old digestive vacuole; white on black point, vesicle formation in zooxanthella's periplast.

and Chia, 1968; Little and Gupta, 1968; Pasteels, 1968; Southward and Southward, 1968). Hence, a similar phenomenon in tridacnids is not novel except possibly in a functional sense. For example, absorption of particulate material by the siphonal surface in tridacnids might contribute directly to the nutrition of the siphon's epidermal cells, but does it necessarily follow that the same function is present in the uptake of fluids?

Zooxanthellae have nutrient salt requirements which might be gratified by means of uptake from the adjacent seawater by the epidermal cells of the hypertrophied siphons. Yonge (1936) has found phosphorus metabolism in *Tridacna crocea* strikingly different from that of the tropical bivalve *Spondylus* in that, not only does *T. crocea* remove significant amounts of phosphorus from its environment, but, unlike *Spondylus,* it also retains the phosphorus excretion products of its own protein catabolism. Yonge attributes these metabolic differences to the demanding nutrient salt requirements of the tridacnid's zooxanthellae. This idea of a strong physiological dependence on phosphorus by zooxanthellae gains additional support from the more contemporary findings of McLaughlin and Zahl (1966) who observe that the population structure of axenically cultured zooxanthellae suffers deleterious effects when grown in culture medium which is phosphate depleted.

What, then, might be the pathway of phosphorus removal from seawater? In the case of some non-tridacnid bivalves, the majority of nutrient salts are simply drunk and later absorbed through the gut walls (Allen, 1970; Fretter, 1953). However, in comparison to the greater portion of the Bivalvia, the tridacnid alimentary tract is categorically small in relation to its total biomass. This aspect, in addition to the apparent extra phosphorus requirements of its symbiont algae, suggests that most salt uptake must enter from another site. At present, the microvillous epidermis of the hypertrophied siphons is the only tridacnid tissue in external contact which appears to be clearly capable of fulfilling this critical role. Further, in terms of conservation of metabolic energy, the siphonal epidermis would likely be the most direct path to the zooxanthellae for the transport of nutrient salts. In any event, while I suggest the possibility of the microvillous epidermis being envolved in uptake of phosphorus, this is pure speculation which will require the affirmative results of ^{32}P pulse labeling for initial substantiation.

I thank Dr. Philip Helfrich, Director of the Eniwetok Marine Biological Laboratory, for providing logistics and facilities necessary for this work. A portion of the travelling expenses were absorbed by NRC Operating Grant A1427 to Professor G. O. Mackie. Thanks are also due to Sir C. Maurice Yonge for his critical reading of this manuscript.

ADDENDUM (Received August 7, 1971)

I have been recently informed in a personal communication from Sir C. Maurice Yonge of the University of Edinburgh that during the period 1962–63 he and the late Thomas F. Goreau of the University of the West Indies completed a series of experiments with *Tridacna elongata* (= *Tridacna maxima*) utilizing radioactively labeled compounds. The results of this study (shortly to be published in their completed form) revealed that both ^{14}C (presumably in the form of bicarbonate) and tritiated leucine were absorbed through the siphonal surface of *T. elongata,* the latter in great quantities. These data clearly substantiate my interpretation of the ultrastructural features of the tridacnid siphons and that, indeed, material is pinocytosed in prodigious amounts through the microvillous border of the siphonal epidermis. Moreover, Johannes (1967), whose study site was within several yards of where I collected the tridacnids used in this paper, has observed that coral reef waters transport organic nutrient material across the leeward inner reef edge accounting for nearly 2% of the total reef community productivity. Hence, it is apparent that adequate nutrient material is available *in situ* which could be absorbed through the tridacnid siphonal epidermis. These observations possibly weaken Yonge's (1953) thesis that the hypertrophied condition of tridacnid siphons evolved *directly* from this bivalve's association with zooxanthellae.

SUMMARY

The question of utilization of zooxanthellae as a holozoic food source by their tridacnid clam hosts was explored utilizing techniques of electron microscopy and electron microscopical histochemistry. It is apparent from the results that older or senescent zooxanthellae are selectively culled from the algal population of the mantle edge by amoebocytes and are intracellularly digested via amoebocyte lysosomes both in the circulatory system and the interdiverticular spaces of the digestive gland. This process cannot be considered "farming," as figured by earlier work, but rather the slow systematic removal and utilization of degenerate zooxanthellae from the algal population of the clam's mantle edge.

Electron photomicrographs of the microvillous surface of the hypertrophied siphons of the *Tridacna* revealed extensive pinocytosis of fluid and particulate material from seawater bathing the clam. It is suggested *a priori* that this endocytosed material contributes to the nutrition of the clam.

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GENETIC VARIATION IN THE MARINE ECTOPROCT *SCHIZOPORELLA ERRATA*

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Organic evolution occurs as genetic changes in populations. However, genetic changes in marine invertebrate animals are not always expressed in the observable phenotype over the short time intervals accessible to the biologist. Conspicuous gene-controlled polymorphisms do occur; *e.g.*, well-documented color polymorphisms of the copepod *Tisbe reticulata* (Battaglia, 1958, 1965), species of the isopod *Sphaeroma* (Bouquet, Levi and Teissier, 1951; West, 1964; Bishop, 1969), and the ascidian *Botryllus schlosseri* (Sabaddin, 1959; Milkman, 1967). But in the vast majority of the 250,000 described species of marine invertebrates characters in the external phenotype controlled by single genes have not been recognized. Thus the study of such material has not substantially contributed to a broad knowledge of the genetic differentiation of marine species. Information on the genetic composition of marine invertebrate species in relation to population size and age structure, mode of reproduction, and ecological factors is just beginning to be obtained.

Recently several studies have been published based on the premise that marker genes useful in genetic studies of marine species can be identified independent of external phenotype, and of breeding experiments, by analysis of variations in proteins (Gooch and Schopf, 1969, 1970; Schopf and Gooch, 1970, 1971, using *Schizoporella errata* and other species of marine ectroprocts; Selander, Yang, Lewontin, and Johnson, 1970, using the arthropod *Limulus;* Manwell and Baker, 1970, using the polychaete *Hyalinoecia* and the pogonophoran *Siboglinum;* and Milkman and Beaty, 1970, using the clam *Mytilus*). This approach utilizes zone electrophoresis on cellulose acetate, starch, or acrylamide media followed by staining for specific proteins. Genetic variability is visualized as variation in protein mobility on the electrophoresis medium. With the use of favorable material genotype and allele frequencies at some polymorphic loci are directly ascertainable.

When a local marine population is sampled, it cannot be assumed that genotypes of a locus will be randomly distributed over the area. Differing fitness values of genotypes in different regimes of current energy, depth, substrate, exposure to light, or biotic association may lead to non-random distributions. Since the larvae of many species remain only hours in the water column, larval settlement in close proximity to parental colonies may yield clumps of like genotypes. Positive assortive fertilization could also lead to this type of clustering.

Schizoporella errata (Fig. 1) is one of the best characterized marine species from a genetic point of view. It is a typical encrusting ectoproct, one of the 3500 living species of the phylum. The brick-red, sessile colonies consist of hundreds to a few thousands of individuals of hermaphroditic yet outbreeding zooids. It is important to emphasize that the larva of this species (and of most ectoprocts) is short-lived (hours) thus greatly limiting the range of gene flow in any single generation. The North American range along the Atlantic Ocean is reportedly

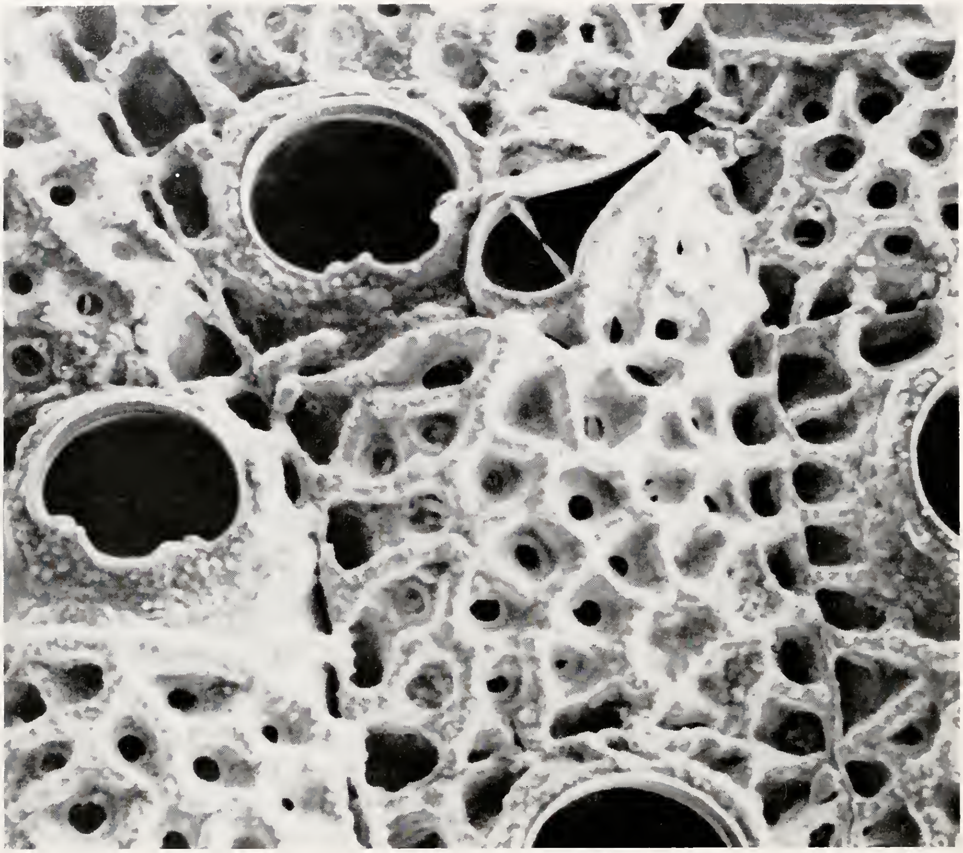

FIGURE 1. Scanning electron microscope photograph of avicularia-bearing individual of a colony of *Schizoporella errata* from Green Pond, Massachusetts. The length of the individual is about 0.6 mm.

from Canada to Florida. *Schizoporella errata* in this paper is apparently the same as *S. unicornis* of all previous literature of the Woods Hole area. Reasons for this purely nomenclatorial change are cited in the detailed descriptions referring to relevant type material and other North American occurrences (Hastings, 1968; Powell, 1970). For purposes of positive identification of the species used in this and previous work, we illustrate here (Fig. 1) a typical specimen. Note the rectangular shape of the zooid, the well-defined sinus of the orifice, the thickened ridges between pores of the frontal surface and the extended (not sharply triangular) avicularium lateral to the orifice.

The fullest interpretation of the data reported here must await a more complete understanding of the size of local populations (now estimated as a few to several hundred colonies extending over tens of m^2), the effect of yearly recruitment (probably substantial at some investigated localities but not at others), and the amount of genetic variability in the rest of the genome. Meanwhile, we wish to present available information and their implications. Results are based on field collections made in the summer of 1969 (reported in Gooch and Schopf, 1970) and 1970 (presented below).

MATERIALS AND METHODS

From 13 to 47 colonies of *Schizoporella errata* were collected from pilings, floating docks, and rock jetties at each of 9 stations (Fig. 3) between Cape Cod, Massachusetts, and Beaufort, North Carolina, during the summer of 1970. Except at Cape Cod Canal and Indian River Inlet, the positions of colonies were mapped *in situ* prior to collecting. The majority of collections were from vertical pilings at the localities near Woods Hole. Pilings were classified as "exposed" or "unexposed" depending upon whether they confronted the full force of currents or were shielded from the force of currents by other pilings, rocks, or the local configuration of the sea shore. Furthermore, exposed pilings were partitioned into "protected" and "unprotected" sides by field determination of the direction of principal currents. Colonies were obtained from 0.5 to 3.0 m below low tide as indicated by the barnacle-line.

Collections awaiting electrophoresis were maintained alive in running seawater or were frozen at −60° C for up to three weeks. Freezing had no discernible effect on enzyme patterns. Material was electrophoresed on acrylamide gel using apparatus manufactured by E. C. Corporation (Philadelphia, Pennsylvania).

Procedures for electrophoresing and staining esterases, malate dehydrogenase, and "leucine" aminopeptidase were as previously described (Gooch and Schopf, 1970). Two new enzymes, alkaline phosphatase and tetrazolium oxidase, have been added to systems under analysis. Electrophoresis for alkaline phosphatase was conducted in 0.09 M Tris and 0.015 M boric acid buffer, pH 8.9, for $2\frac{1}{2}$ hours. Gels were incubated in 125 ml 0.04 M Tris and 0.048 N HCl adjusted to pH 7.2 for 3 hours at room temperature. The staining mixture consisted of 50 mg sodium alpha-napthyl phosphate, 50 mg Fast Blue RR Salt, 350 mg polyvinylpyrolidone, 100 mg NaCl, and 0.1 ml of 10 per cent $MgCl_2$. The technique for staining tetrazolium oxidase is similar to that for malate dehydrogenase (Gooch and Schopf, 1970) except that substrate was omitted and development took place in full light. Formazan salts densely stain the gel in areas not occupied by tetrazolium oxidase.

Throughout the Cape Cod to Beaufort transect, an aggregate of 250 colonies of *S. errata* were analyzed for "leucine" aminopeptidase, 91 colonies for esterases, 49 for alkaline phosphatase (Green Pond and Duke Marine Laboratory not sampled), and 18 for tetrazolium oxidase (data for Cuttyhunk, Indian River Inlet, and Shark Shoal Jetty).

The methodology of identifying genetic loci in the absence of breeding tests parallels the approach used by Selander, Yang, Lewontin, and Johnson (1970) for work on *Limulus*, and has been extensively discussed previously (Gooch and Schopf, 1970).

To facilitate statistical treatment of material from localities in the Woods Hole region, adjacent collecting stations that do not show significant differences in gene frequencies were pooled. Thus, as is shown later, the Marine Biological Laboratory (MBL) dock and Sheep Pen Harbor populations (74 colonies) were pooled, as were populations from Robinsons Hole and Cuttyhunk (66 colonies).

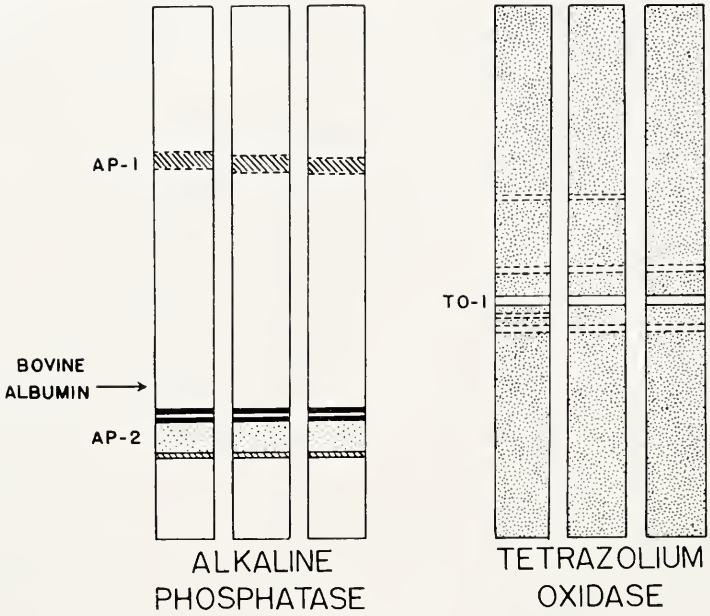

FIGURE 2. Diagram illustrating patterns of banding in *Schizoporella errata* for alkaline phosphatase and tetrazolium oxidase. For alkaline phosphatase, dark bands represent heavily-stained zones of enzyme activity, hatched bands stand for more lightly stained zones, and stippled bands represent faint zones. For tetrazolium oxidase, the clear band represents no staining, and dashed bands represent light staining.

RESULTS

New gene loci

Characteristics of 8 loci were previously documented (Gooch and Schopf, 1970) and these are not different in the material discussed here. Three additional loci, 2 governing alkaline phosphatase patterns, and 1 for tetrazolium oxidase, are newly defined (Table I), making a total of 11 loci characterized for *Schizoporella errata*.

Alkaline phosphatase. Alkaline phosphatase stains as two band systems on acrylamide gel. The enzyme band of the locus Ap-1 averages 0.42 in mobility relative to a Bovine serum albumin (Nutritional Biochemicals Corp.). Resolution in buffer systems tried to date is too poor to establish whether the locus is polymorphic or not.

The second locus, Ap-2, yields 3 closely-spaced and well-resolved bands (Fig. 2). It is monomorphic in all sampled populations for the same allele, designated as Ap-$2^{1.10}$.

Tetrazolium oxidase. Distinct zones that do not precipitate formazan staining from the decomposition of tetrazolium salts in strong light are often designated as "achromatic zones." These zones have the character of indophenol oxidase in human erythrocytes (Brewer, 1967). The biochemical properties and *in vivo* catalytic activities of the enzyme giving rise to achromatic zones in *S. errata* have not been investigated; we therefore prefer the operational term "tetrazolium oxidase," following Baur and Schorr (1969).

There is a single zone of tetrazolium oxidase activity, the product of the locus To-1 (Fig. 2). The zone consists of one major band and 2–4 faint leading and trailing bands. Uniform mobility in all populations sampled is indicative of monomorphism for a single allele, here designated To-$1^{0.78}$.

Local distribution of gene frequencies and genotypes at the Lap-3 locus

The Lap-3 locus is an autosomal gene segregating for two codominant alleles, Lap-$3^{0.94}$ and Lap-$3^{0.98}$ in populations near Woods Hole (Gooch and Schopf, 1970). Since it is the sole polymorphic locus in the majority of sampled populations, it is uniquely suitable for analysis of allele frequencies and genotype distribution in local populations.

Effect of depth. Depth is a negligible factor at Green Pond, where most colonies were taken from a floating dock. In the pooled MBL-Sheep Pen Harbor populations 39 colonies were obtained within 1 m of the low tide line, and 35 colonies in the next 2 meters. Gene frequency does not differ significantly in depth comparisons (0.94 allele = 0.55 at 0–1 m, 0.63 at 1–3 m; $\chi^2 = 0.9$, $P > 0.30$). There is, however, a significant excess of heterozygotes in the 0–1 m interval (27 of 39 colonies; $\chi^2 = 6.2$, $P < 0.02$). Genotype distribution between 1–3 m agrees with a Hardy-Weinberg distribution ($\chi^2 = 0.02$, $P > 0.80$).

Since no colonies in the pooled Robinsons Hole-Cuttyhunk populations were taken above 1 m, comparisons are drawn between 1–1.5 m (48 colonies), and 1.5–3.0 m (17 colonies); (one colony on a nearby rock not included). Gene frequency is nearly identical in both depth zones (0.94 allele = 0.32) and genotype distribution in both zones conforms to Hardy-Weinberg equilibrium (1.0–1.5 m, $\chi^2 = 1.9$, $P > 0.10$; 1.5–3.0 m, $\chi^2 = 1.3$, $p > 0.30$).

In 1969 collections, the 0.98/0.98 homozygotes appeared to increase with depth (Gooch and Schopf, 1969, Fig. 5) at the pilings of the MBL dock. Two of the 4 homozygotes occurred at depth less than 2 m (n = 36), and 2 occurred at depth greater than 2 m (n = 4). In 1970 collections, 3 of the 4 homozygotes occurred at depths less than 2 m (n = 41), and 1 occurred at depths greater than 2m (n = 4). If it is assumed that the population has remained uniform genetically both years the data may be pooled to give a significant excess of 0.98/0.98 homozygotes in the deeper water. (2 × 2 table, Yates' correction; $\chi^2 = 4.9$, $P < 0.05$).

Effect of exposure to wave action. The pooled MBL dock-Sheep Pen Harbor populations contained 38 colonies on exposed pilings, 20 protected and 18 unprotected, and 36 colonies on unexposed pilings. Pooled Robinsons Hole-Cuttyhunk populations consisted of 52 colonies on exposed pilings, 31 protected and 21 unprotected, and 14 on unexposed pilings. There are no significant differences in gene frequencies in comparisons of exposed and unexposed pilings (MBL dock-Sheep Pen Harbor, $\chi^2 = 0.06$, $P > 0.80$; Robinsons Hole-Cuttyhunk, $\chi^2 = 0.16$, $P > 0.60$). Nor are there any significant differences in comparisons of protected and unprotected sides (MBL dock-Sheep Pen Harbor $\chi^2 = 0.1$, $P > 0.80$; Robinsons Hole-Cuttyhunk, $\chi^2 = 1.3$, $P > 0.20$).

Genotype distributions on exposed versus unexposed pilings, and protected versus unprotected sides of pilings were compared with expected Hardy-Weinberg genotype distributions. All comparisons are consistent with Hardy-Weinberg values except unexposed pilings at MBL dock-Sheep Pen Harbor, where the heterozygote class is predominant ($\chi^2 = 6.4$, $P < 0.02$), and the protected sides of exposed pilings at Robinsons Hole-Cuttyhunk, where the heterozygote class is significantly small ($\chi^2 = 4.6$, $P < 0.05$).

Effect of time. Two localities, Green Pond and the MBL dock, were mapped and sampled during both 1969 and 1970 (Fig. 4). Gene frequency at Green Pond for the 0.94 allele was 0.76 ($n = 43$) in 1969 and also 0.76 ($n = 47$) in 1970. At the MBL dock, the frequency of the 0.94 allele was 0.72 ($n = 50$) in 1969 and 0.61 ($n = 45$) in 1970. The difference is not significant ($\chi^2 = 2.5$, $P > 0.15$). Thus no significant temporal change in over-all gene frequency has been detected.

The 1969 collections yielded evidence of genotypic differences at Green Pond (Gooch and Schopf, 1970). The boat mooring side of the floating dock was occupied by a significant excess of heterozygotes compared to the less polluted open harbor side ($\chi^2 = 4.3$, $P < 0.05$). In the 1970 sample, only 8 colonies were obtained from the mooring side. As in 1969, there was an excess of heterozygotes on the mooring side, with 37 per cent heterozygous colonies as compared with 22 per cent heterozygotes on the open harbor side (Fig. 4). However, the difference in 1970 does not approach statistical significance (homozygous classes combined in 2 × 2 table, $\chi^2 = 0.7$, $P > 0.30$).

Genetic variation in S. errata

Table I lists 11 defined loci. Eight are well-established as predominantly monomorphic in populations and one, Lap-3, as chiefly polymorphic. The remaining 2 loci are not usable in all populations; Ap-1 because band variation is not interpretable, and Lap-2 because the protein often stains too faintly for analysis. Mobility variation for the E-3 locus in the Cape Cod Canal population is strongly

TABLE I

Gene loci defined in Schizoporella errata *together with allele nomenclature for populations sampled in the Virginian Faunal Province. Note that E-3 has 2 alleles at the Cape Cod Canal (southern border of Acadian Fauna Province), and that Lap-3 has 1 allele in the Carolinian Faunal Province*

Enzyme system	Locus	Number of alleles	Designation of alleles
(1.) Esterase	E-1	1	E-1$^{.10}$
	E-2	1	E-2$^{.66}$
	E-3	1	E-3$^{.75}$
	E-4	1	E-4$^{1.18}$
(2.) Malate dehydrogenase	M-1	1	M-1$^{.99}$
(3.) "Leucine" aminopeptidase	Lap-1	1	Lap-1$^{.31}$
	Lap-2	1	Lap-2$^{.68}$
	Lap-3	2	Lap-3$^{.94}$
			Lap-3$^{.98}$
(4.) Alkaline phosphatase	Ap-1	1–2 (?)	Ap-1$^{.42}$
	Ap-2	1	Ap-2$^{1.10}$
(5.) Tetrazolium oxidase	To-1	1	To-1$^{.78}$

Table II

Summary of gene and genotype distributions for the Lap-3 locus for Schizoporella errata. *MBL means Marine Biological Laboratory; DML means Duke Marine Laboratory*

Locality	Number of colonies	Gene frequency		Genotype (N)		
		Low mobility allele	High mobility allele	Low mobility homozygotes	Hetero-zygotes	High mobility homozygotes
Cape Cod Canal	15	0.80	0.20	10	4	1
Green Pond	47	0.76	0.24	28	15	4
MBL Dock	45	0.61	0.39	14	27	4
Sheep Pen Harbor	29	0.62	0.38	10	16	3
Robinsons Hole	36	0.31	0.69	4	14	18
Cuttyhunk	30	0.35	0.65	6	9	15
Indian River Inlet	13	0.81	0.19	8	5	0
DML Dock	15	1.00	0.0	15	0	0
Shark Shoal Jetty	20	1.00	0.0	20	0	0

suggestive of a second polymorphism. Since this locus shows no variability in the other populations it is assigned overall as monomorphic.

Thus the amount of polymorphism stands at 1 of 9 well-established loci (11 per cent) in the majority of populations and 2 of 9 (22 per cent) in the Cape Cod Canal population. In a few Cape Cod populations Lap-2 is distinguishable as monomorphic. There 1 of 10 (10 per cent) loci are polymorphic.

Regional genetic variation

Throughout the Cape Cod to Beaufort transect 7 loci are uniformly monomorphic for the same alleles. The E-3 locus, as explained above, appears to be polymorphic in the Cape Cod Canal population and is definitely monomorphic in other populations.

The general low level of genetic variation contrasts with the Lap-3 locus, which is polymorphic for the 0.94 and 0.98 alleles in every sampled population north of Cape Hatteras (Table II). Allele frequencies vary widely in segregating populations. The 0.98 allele increases from 0.24 at Green Pond to 0.69 at Robinsons Hole paralleling a local southwestward cooling of summer water temperatures. Regionally, the 0.98 allele decreases from 0.39 at the MBL dock to 0.19 at Indian River Inlet and to 0.0 at DML dock and Shark Shoal Jetty (Fig. 3). This parallels a regional increase in summer water temperatures. Both trends, as well as the increase in the frequency of 0.98/0.98 homozygotes in the deeper, cooler water at MBL dock, suggest selection against the 0.98 allele under warmer conditions. Koehn (1969, 1970) attributed a clinal distribution of allele frequencies at an esterase locus in Catostomid fishes to temperature dependent differences in enzyme activity. Details of the Green Pond-Cuttyhunk transect are presented elsewhere (Schopf and Gooch, 1971).

The Lap-3$^{0.98}$ allele has a frequency of 0.20 in the sampled population from Cape Cod Canal. Animals living in the canal are subject to *daily* fluctuations in temperature of up to 11° C in the late summer (when the material was collected). This remarkably large daily temperature change is due to the water in

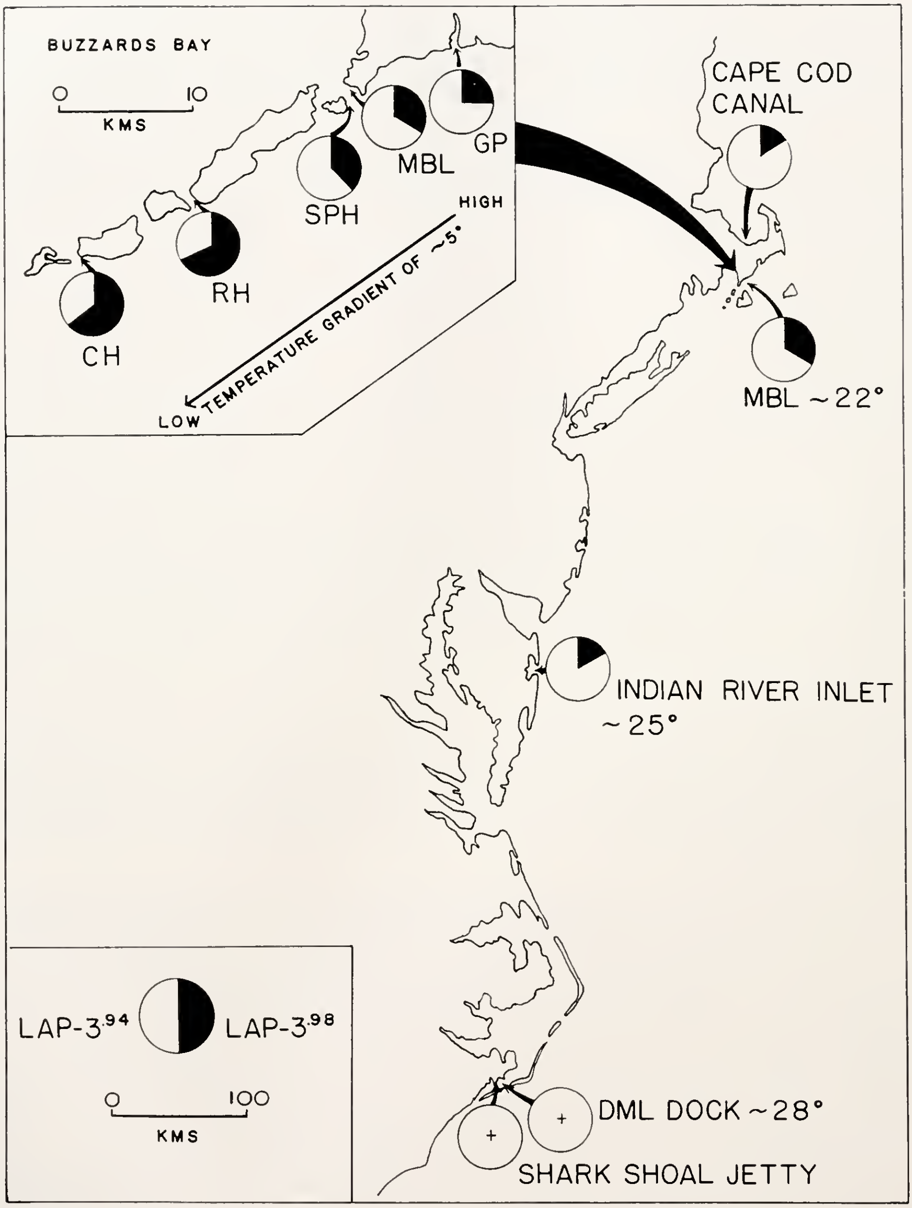
BUZZARDS BAY
0
10
KMS
CH
RH
SPH
MBL
GP
HIGH
LOW
TEMPERATURE GRADIENT OF ~5°
CAPE COD CANAL
MBL ~22°
INDIAN RIVER INLET ~25°
LAP-3.94
LAP-3.98
0
100
KMS
DML DOCK ~28°
SHARK SHOAL JETTY

FIGURE 3. Map showing location of 9 collecting localities together with gene frequencies for Lap-3 alleles. Temperatures cited are for the warmest time of the year (late August), and are chiefly meant to emphasize the trend in water temperatures and the relative difference between localities. Note that the frequency of the Lap-$3^{0.98}$ allele decreases as temperature increases in both the Cuttyhunk (CH) to Green Pond (GP) transect and the coastal transect. See text for further discussion. MBL stands for Marine Biological Laboratory dock; SPH for Sheep Pen Harbor; RH for Robinsons Hole; DML for Duke Marine Laboratory.

the canal alternately filling with cold water from Cape Cod Bay and warmer water from Buzzards Bay, depending upon the tidal phase (Administrative-Technical Advisory Committee, 1968; Fairbanks, Collins and Sides, 1968). *S. errata* is apparently dormant during the winter when, in fact, large temperature differences do not exist between these bodies of water. Differential selection with respect to temperature or correlative factors would appear to operate on the Lap-3 locus only during the highest environmental temperatures which are typical of late summer. Thus it is not surprising that allele frequencies indicate selection for individuals able to live in very warm water even though the locality is on the southern edge of the Acadian Faunal Province.

Discussion

Local distributions of allele frequencies and genotypes

The majority of tests of gene and genotype variation with depth, current exposure, and time have revealed no evidence for non-random or temporally changing distributions. Of 17 local distributions 4 have been found which differ significantly from expected values. There was (1) an excess of heterozygotes in the upper 1 m at MBL dock-Sheep Pen Harbor, (2) an excess of the 0.98/0.98 homozygote class in deeper sampling of the MBL dock-Sheep Pen Harbor pilings, (3) an excess of heterozygotes on unexposed pilings at MBL dock-Sheep Pen Harbor, and (4) a deficiency of heterozygotes on the protected sides of exposed pilings at Robinson Hole-Cuttyhunk. It should be remarked that deviations from expected values never strikingly large (P always greater than 0.01).

The excess of heterozygotes in shallowest water may indicate that heterozygotes have superior tolerance to the relatively more variable zone just below the surface. A heterozygote advantage is also a possible explanation for the heterozygote excess found in 1969 at Green Pond on the side of the floating dock nearest the boat moorings (Gooch and Schopf, 1970).

The excess of the Lap-$3^{0.98}$ allele with depth is interpretable in terms of selection for this allele in cooler (deeper) water. However this association with temperature is much more convincingly developed with the local and regional distribution of the Lap-3 alleles. We presently have no evidence that temperature, itself, rather than an unknown environmental factor operating in a temperature-dependent manner, is the direct agent of selection.

The excess of heterozygotes on unexposed pilings, and the deficiency of heterozygotes on protected sides of exposed pilings appear to be contradictory results. We cannot explain these observations based on present information.

Regional genetic variation

The general outlines of the regional genetic differentiation of *Schizoporella errata* can now be drawn. Nine well-characterized gene loci were surveyed in a transect which includes 3 zoogeographic provinces (Johnson, 1934; Cerame-Vivas and Gray, 1966). The Cape Cod Canal population borders the Boreal or Acadian Province, the remaining Cape Cod populations and the Indian River Inlet population are located in the Virginian Province, and the Beaufort populations are in the Carolinian Province. The estimated number of polymorphic loci declines

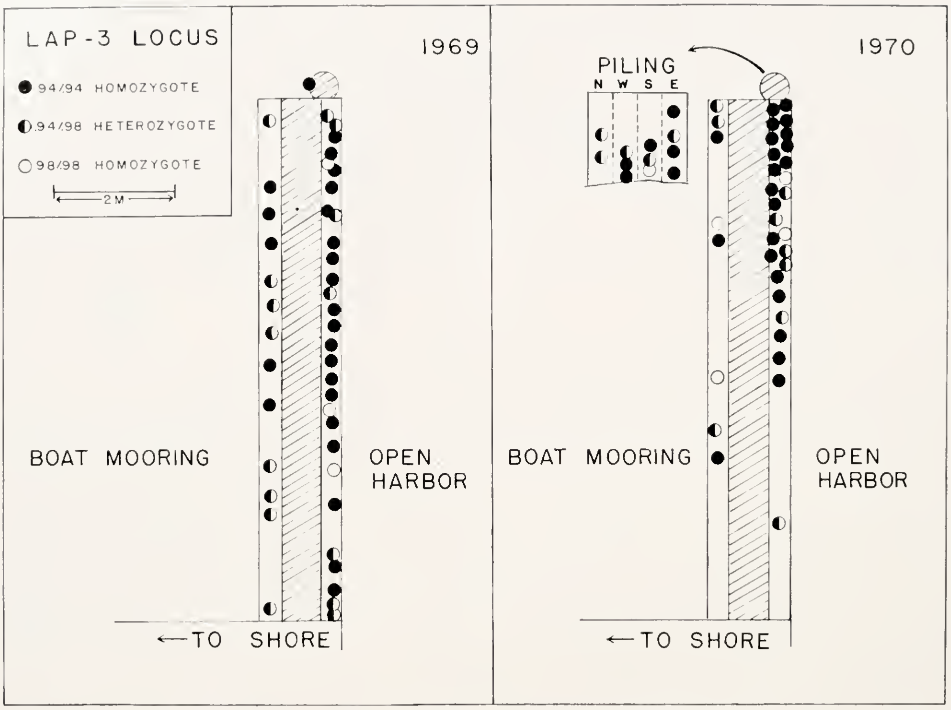

FIGURE 4. Distribution of genotypes of "leucine" aminopeptidase (locus Lap-3) in colonies of *Schizoporella errata* occurring on outer floating dock of boat moorings at Green Pond, Massachusetts. White area on floating dock represents area available for colonization.

southward from 2 at Cape Cod Canal (Lap-3 and E-3) to 1 (Lap-3) in populations of the Virginian Province, and 0 in the populations of the Carolinian Province. Samples are small and data from additional protein systems (and more loci) are much needed to determine if southward diminution of genetic variability is real.

On a regional scale, two major conclusions follow from the pattern of gene frequencies at the diallelic Lap-3 locus: (1) the geographic scale of genetic differentiation may be only a few km since populations separated by 13 km can differ significantly in allele frequency (this aspect is explored fully for populations in the vicinity of Woods Hole in Schopf and Gooch, 1971); and (2) the trend of allele frequencies from Cape Cod to Beaufort approximates a cline, suggesting a regional pattern of natural selection rather than control of gene frequencies by purely local selection pressures or random drift.

On the other hand, the uniform monomorphism of 7 loci throughout the transect, and 8 south of the Cape Cod Canal, is a striking feature of the genome of *S. errata*. Thus 82–89 per cent of the sampled genome is without intra- and interpopulation genetic variation. The occurrence of the same "best allele" at the majority of sampled loci in populations from 3 zoogeographic provinces suggests that *S. errata* is a genetically close knit species, and that adaptation may be primarily regional rather then local. Apparently single alleles have arisen at a substantial number of loci whose protein products function effectively under the varied conditions throughout the coastal transect. This lack of genetic variation is all the more interesting, since the very limited larval life suggests little gene flow along the full range of the species.

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SUMMARY

Schizoporella errata is sessile and has larvae that live only hours. On a purely local scale, the spatial distribution of gene frequencies and genotypes appears random in most comparisons. In addition, genotypes and gene frequencies remained relatively stable after the passage of a year. Over a distance of 1000 km from the southern edge of the Acadian Faunal Province through the Virginian Faunal Province and into the Carolinian Faunal Province, from 80 to 89 per cent of the sampled genome is identical. That is, genetic polymorphism stands at 1 of the 9 well-established loci (11 per cent) and an estimated 2 of 10 total loci in pooled material from 9 localities. The proportion of alleles at the single, clearly polymorphic locus (Lap-3) varies directly as a function of environmental temperature measured at the warmest time of the year.

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REEF CORALS: AUTOTROPHS OR HETEROTROPHS?

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Some recent studies[2] seem to indicate that the nutritional economy of reef corals is for all practical purposes to be considered autotrophic due to their zooxanthellae (Fig. 1). For example, Franzisket (1969a, 1970) claims to have demonstrated that some Hawaiian reef corals can achieve net growth in the total absence of particulate food, while Johannes and Coles (1969) state that the energy requirements of Bermudian reef corals are in some cases more than an order of magnitude greater than could be provided by the zooplankton which the investigators were able to catch with a fine net.

In spite of their supposedly autotrophic economy, the reef corals have not developed any of the behavioral and structural specializations for such a way of life. In this respect they differ fundamentally from *Xenia hicksoni* and *Clavularia hamra* (Octocorallia, Alcyonacea) (Gohar, 1940, 1948) and *Zoanthus sociatus* (Hexacorallia, Zoanthidea) (Von Holt and Von Holt, 1968a, b), unrelated anthozoans which have independently evolved a more or less complete nutritional dependence upon their contained zooxanthellae. Available data is summarized in Table I. These species have never been observed to feed, and there is a more or less marked reduction of structures and functions associated with the usual predatory feeding habits in Cnidaria; for example, they do not respond to any of the known tactile and chemical stimuli that trigger feeding behavior in related carnivorous species; they do not ingest particulate matter, and are unable to either digest or assimilate food artificially placed into their coelenteron by means of a canula (Goreau and Goreau, unpublished).

The reef corals are, by contrast, superbly efficient and voracious carnivores that will accept practically any kind of particulate animal food (Yonge, 1930a, 1930b; Yonge and Nicholls 1930, 1931). Feeding occurs in several different ways, depending on the species: in the majority, the food is swept into the coelenteron by means of ciliary currents, (sometimes involving reversal as in *Fungia*), while in some corals the tentacles convey the food directly to the mouth (Yonge, 1930a). Most species are also capable of extracoelenteric digestion of food matter outside the body by means of mesenterial filaments extruded through temporary openings (Fig. 2) at any place on the colony surface (Duerden, 1902; Matthai, 1918; Goreau, 1956). Reef corals obtain food via this ancillary route and also use the extruded filaments as weapons, primarily against other corals the tissues of which they may digest (Lang, 1969, 1970).

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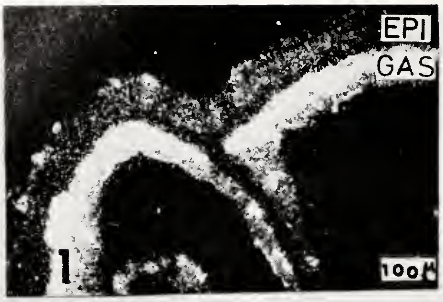
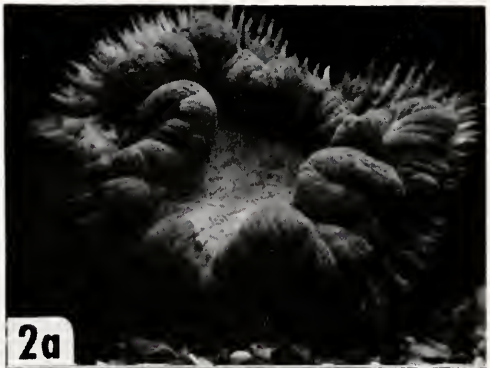
[2] See also the paper by V. B. Pearse and L. Muscatine (dedicated to the late T. F. Goreau) on pages 350–363, and that by P. V. Fankboner on pages 222–234 of this issue—*Editor.*





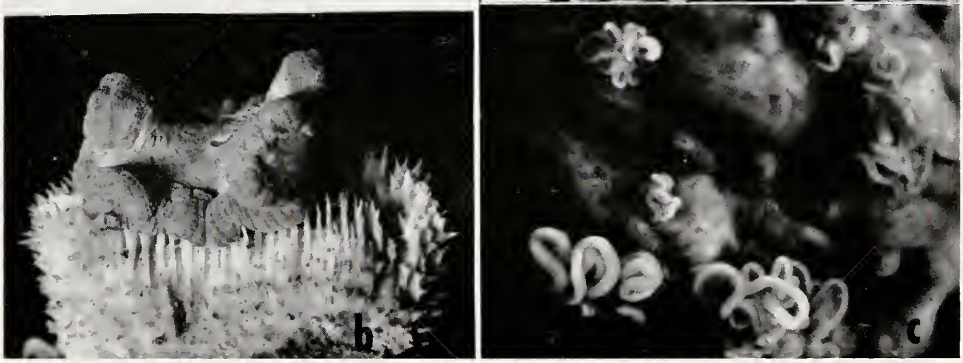


FIGURE 1. Autoradiogram of carbon^{14} labelled *Fungia scutaria.* The coral was exposed to $^{14}CO_2$ in sunlight for ten minutes and washed in running sea water for thirty minutes before fixation. The area seen under dark field illumination and focused on the plane of emulsion shows the concentration of silver grains over the carbon^{14} labelled zooxanthellae of the gastrodermis. The scale represents 100 μ. Specimens exposed in the dark showed no fixation of the isotope.

FIGURE 2. *Mussa angulosa* under severe starvation gradually loses contact with the skeleton (a) (b), eventually sinking to the bottom of the aquarium, still alive. When offered crab juice this free specimen extruded its mesenterial filaments (c) through the epithelium of the calicoblast. Some colonies lose their zooxanthellae, some keep them, but in the most severe cases of starvation only the stomodeum and a few filaments remain. Later only bits of mesenterial filaments curl about. However they all showed feeding responses when crab meat or amino acids were added. These filaments persist for a few days.

These diverse feeding mechanisms are supplemented by an exquisitely perceptive chemotactic sense. In several species of Jamaican reef corals (*Manicina areolata, Cladocora arbuscula, Eusmilia fastigiata, Isophyllia sinuosa, Mussa angulosa* and *Scolymia lacera*) we found some years ago that very low concentrations of amino acids such as glycine, alanine, phenylalanine and lucine could trigger off typical feeding responses; *i.e.*, opening and eversion of the stomodeum, swelling of the coenosarc, extension of tentacles and sometimes extrusion of mesenterial filaments. *M. areolata* responded in this manner to alanine and glycine at concentrations as low as 10^{-9} M, whereas glucose, sucrose, glycerol and mannitol did not have any effect at high concentrations. Mariscal and Lenhoff (1968) ob-

Table I

Available data on nutritional adaptation and absorption in Scleractinia, Alcyonacea and Zoanthidea

Taxonomy	Zooxanthellae	Absorption of crab juice and india ink into filaments	Uptake of ^{3}H leucine into the epidermis	Uptake of ^{14}C by zooxanthellae	Feeding response to crab meat or amino acids	Nematocysts
Scleractinia:						
Hermatypes:						
Fungia	+	+++	+++	++	+++	+++
Stylophora	+	+++	+++	++	+++	+++
Ahermatype:						
Tubastrea	0	+++	+++	0	+++	+++
Alcyonacea:						
Xenia	+	0	?	+++	0	0
Zoanthidea:						
Z. sociatus	+	?	?	+++	?	disordered
P. caribbae	+	+++	?	+	+++	++
P. grandis	+	+++	?	+	+++	++

Other morphological correllates with xanthellar symbiosis are:

(1) Xenia: *no nematocysts, reduced filaments, no septal lobes,*

(2) Zoanthus: *nematocysts, but these are in a disordered position and in places where they do no good; filaments reduced but lobes are very large. All stages of pycnosis, degeneration, fragmentation and extrusion of zooxanthellae were observed in the mesenterial lobes.*

Feeding reaction:

(1) *Corals:* *Dilation and extension of stomodeum, imbibition of water, sometimes erection of tentacles or shooting of the mesenteries through mouth or body wall*;

(2) *Xeniids:* *Rhythmic movement of tentacles of anthocodia*;

(3) *Zoanthids:* Zoanthus sociatus: *none,*
Palythoa caribbae: *Dilation of mouth, strongly inward movement of water at ciliate groove, curling over of the tentacular rim,*
Palythoa grandis: *same as* P. caribbae.

served that concentrations as low as 10^{-7} M proline resulted in feeding responses in *Cyphastrea ocellina*, *Pocillopora damicornis* and *Fungia scutaria*.

Responses similar to those caused by amino acids are produced in corals by seawater in which there had previously been zooplankton. We have often observed that corals will expand under natural conditions in apparent anticipation of plankton; evidently this is due to their ability to sense the diffuse cloud of metabolites, including amino acids, that usually surrounds plankton swarms (Hellebust, 1965). It would indeed be surprising if, as Johannes and Coles (1969) have speculated, the corals have retained these capabilities merely to obtain trace nutrients such as phosphorus from their prey while the bulk of their nutrition comes from the zooxanthellae! Yet, there is no need for such a roundabout way to obtain phosphorus since reef corals, but *not* ahermatypes, in the light are known to take up inorganic phosphate from the medium, this being a function of the zooxanthellae, not the coral host (Yonge and Nicholls, 1931). The fully autotrophic xeniid alcyonaceans and *Zoanthus* are evidently able to obtain all their trace nutrients directly from the seawater. As regards the possible need for organic phosphorus, Von Holt (1968) has shown that in *Zoanthus* there is a transfer of nucleoside polyphosphate from algal symbiont to animal host. If the reef corals were truly as autotrophic as Franzisket and Johannes believe, the question arises why have they not evolved similar more direct mechanisms for obtaining critical nutrients directly from their symbionts?

OBSERVATIONS

The boundary layer water and its relation to the trophic structure of the reef

The evidence so far cited has not resolved the conflict between the apparent low productivity of tropical ocean surface waters (Fleming, 1954; Sargent and Austin, 1949, 1954) and the need for organic nutrients by the benthonic fauna in the reef ecosystem. This consists of the corals and a diverse assemblage of filter, detritus, suspension and deposit feeders as well as predacious carnivores, the majority without zooxanthellae which might serve as ancillary food source. Recent reviews by Bakus (1969) and Stoddart (1969) have demonstrated how little quantitative information is available on the trophic cycles within the reef biotope, largely because the pathways themselves are still largely unknown. Oceanic reef ecosystems appear on the whole to be autotrophic units operating at very high levels of productivity, turnover rate and efficiency (Odum and Odum, 1955; Kohn and Helfrich, 1957) whereas at least some smaller reefs off high islands may be non-autotrophic (Goreau, Torres, Mas and Ramos, 1960; Gorden and Kelley, 1962). In view of the low trophic potential of the tropical oceanic waters, high localized productivity of reefs can only be achieved through coupled internal recycling systems that reduce external losses of free energy to a minimum and thus maintain the local nutrient levels at high steady state values.

The existence of such internal cycles is reflected in the marked differences that may be observed between the outside ocean water and the water circulating within the reef which will be referred to here as the boundary layer water. Whereas the former is clear and deficient in plankton and other suspended matter, the latter is relatively turbid due to the much higher concentrations of suspended particulates, consisting of both inorganic and organic detritus stirred up by the turbulence, or added to the water by benthonic biota. Near high islands, both particulate and dissolved nutrients in the sea are increased by run-off from the land. The boundary layer water also contains a relatively high concentration of zooplankters, swarms of which shelter and feed within the multitude of crevices and other microhabitats of the reef frame. This environment is extremely difficult to sample quantitatively, but can be readily observed by anyone diving on the reef.

The depauperate and heavily cropped condition of the shallow reef zones described by Bakus (1967, 1969) for some Pacific reefs, and by Johannes and Coles (1969) for Bermuda have been corroborated by the first author's own observations on parts of the Great Barrier Reef, Eniwetok, Saipan and the Red Sea, and is also observed in the shallow reefs of Jamaica and other Caribbean islands. However, conditions in the deeper parts of the outer reef slope vary considerably from extreme impoverishment as for example in Saipan or Eniwetok to a marked increase in species diversity, size and biomass of the macrobenthos, such as is observed in Jamaica (Goreau and Hartman, 1963; Goreau and Wells, 1967). Here the fore reef slope habitat is characterized by very large and diverse standing crops of corals, sponges, Gorgonacea, anemones, Antipatharia, and various algae such as *Halimeda* (Goreau and Graham, 1967); the interstices of the reef frame contain an abundant fauna of Foraminifera, sponges (Hartman and Goreau, 1970), hydrozoans, ahermatypic corals, worms, bivalves, brachiopods (Jackson, Goreau and Hartman, 1970), bryozoans, echinoderms, tunicates and arthropods. Only the hermatypic corals, with the great majority of other coelenterates, contain zooxanthellae, the remainder do not. Above sixty meters the corals predominate, below this the sponges prevail although reef corals occur in diminishing amounts to at least one hundred meters.

The boundary layer water is in continuous and dynamic exchange with the reef biota. We established this by releasing small clouds of India ink from syringes in various microhabitats of the Jamaican fore reef slope at depths of 50 to 60 meters where wave turbulence is low. We found that the India ink was cleared from the water within a few minutes, mostly by the sponges. It appears that a continuous downward flow of particulate matter moves from the boundary layer through the reef, recycling nutrients within the benthos. Quantitative measurements of this exchange have now been carried out *in situ* by H. M. Reiswig (Biology Department, Yale University) in Discovery Bay, Jamaica.

Suspended particulate matter in the reef as a possible food source for corals

The particulate suspended organic matter, organic aggregates and dissolved organic substances circulating in the boundary water of the reef may be of crucial importance to the nutrition of the benthonic fauna, corals included. Marshall (1965) showed that the amount of fine suspended organic detrital matter in the waters of Eniwetok Atoll was between one and two orders of magnitude greater than could be collected with the finest plankton nets. In Jamaica, the macroscopic organic particulates consist chiefly of comminuted vegetable matter, fragmented animal remains of diverse origin, faecal pellets, *etc.*, but we have not yet investigated the much larger microscopic and submicroscopic fractions. Coral-browsing acanthurid and scarid fish contribute large volumes of ground-up carbonates to the suspended matter (Bardach, 1961). During periods of rough weather wave turbulence stirs up fine organic detritus, the leptopel, from the bottom sediment, and clouds of this material roll down over the reef communities of the seaward slope into deep water. At the same time, colloidal and dissolved organic matter are aggregated into larger particles at the surface of bubbles stirred up by the surf (Baylor and Sutcliffe, 1963; Riley, 1963). Mucus is secreted into the water in large amounts by benthonic animals in the reef, chiefly sponges, gorgonians, corals and molluscs (Marshall, 1965). Corals and alcyonarians continuously void large numbers of excess zooxanthellae in strings of mucus (Yonge and Nicholls, 1931). The gonadal products of sponges and echinoids periodically reach such high concentrations as seriously to reduce underwater visibility in the vicinity of the reef.

The question of whether any of these diverse organic particulates are available as food to the corals is still undecided. Part of the difficulty in relating reef corals to their potential food supply is a conceptual one. As the result of Yonge's studies (1940), the corals have been thought of principally as specialized planktivorous carnivores. However, we have numerous observations which seem to indicate that many of the reef corals are not restricted in their feeding to zooplankton since they also seem to feed on any organic particulates that happen to be carried into the coelenteron and from which nutriment may be extracted. In our experience, many reef corals are relatively unspecialized detritus feeders (Fig. 3), capable of utilizing a wide range of organic matter and bacteria (R. A. Kinzie III, Department of Zoology, University of Georgia, personal communication). An example of this is the common Indo-Pacific reef coral *Fungia scutaria* (Goreau, Goreau, Yonge and Neumann, 1970) although it is not yet known what part of its total energy requirements are met from exogenous particulates other than zooplankton.

The uptake of dissolved organic matter by corals

We have not so far considered the possibility of direct utilization of dissolved or colloidal organic matter by scleractinian corals. No attempt will be made here to answer the question of whether there is enough dissolved organic matter of the right kind circulating within the reef to be a significant source of energy for corals; rather we wish to point out that the corals have highly developed structural and functional adaptations for the absorption of dissolved organic matter directly from the sea, and that they can take up compounds such as amino acids

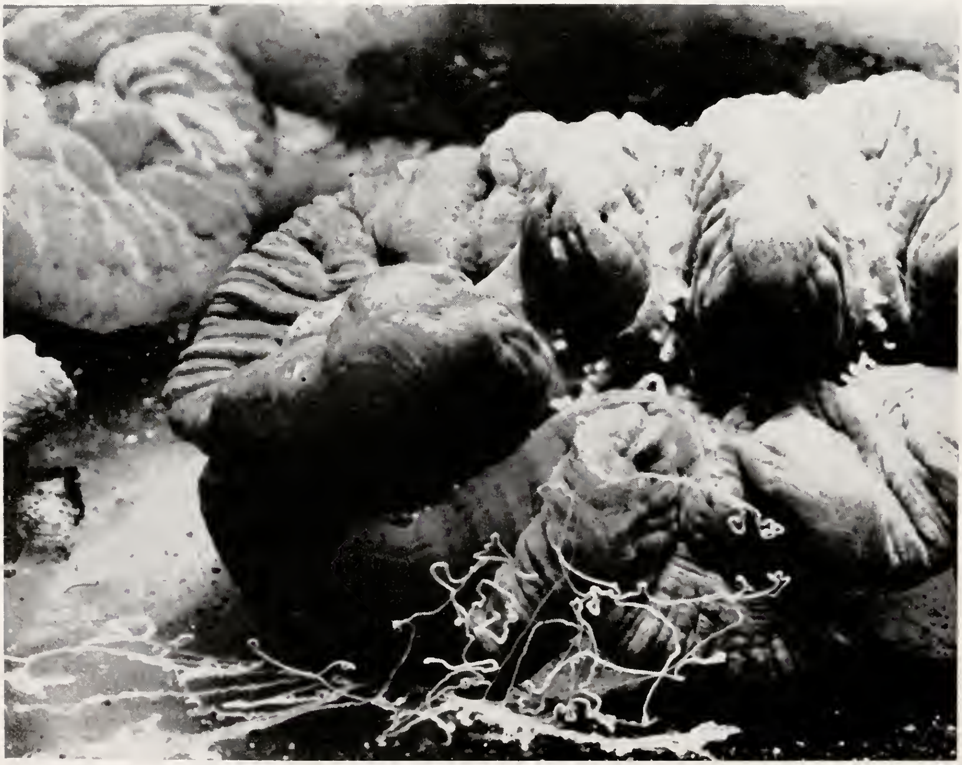


FIGURE 3. *Meandrina meandrites* f. *danae* behaving as a detritus feeder is seen here sweeping the mud bottom with huge loops of mesenterial filaments extruded through the column wall after crab extract has been added to the media. These corals also showed feeding reactions when offered alanine and glycine.

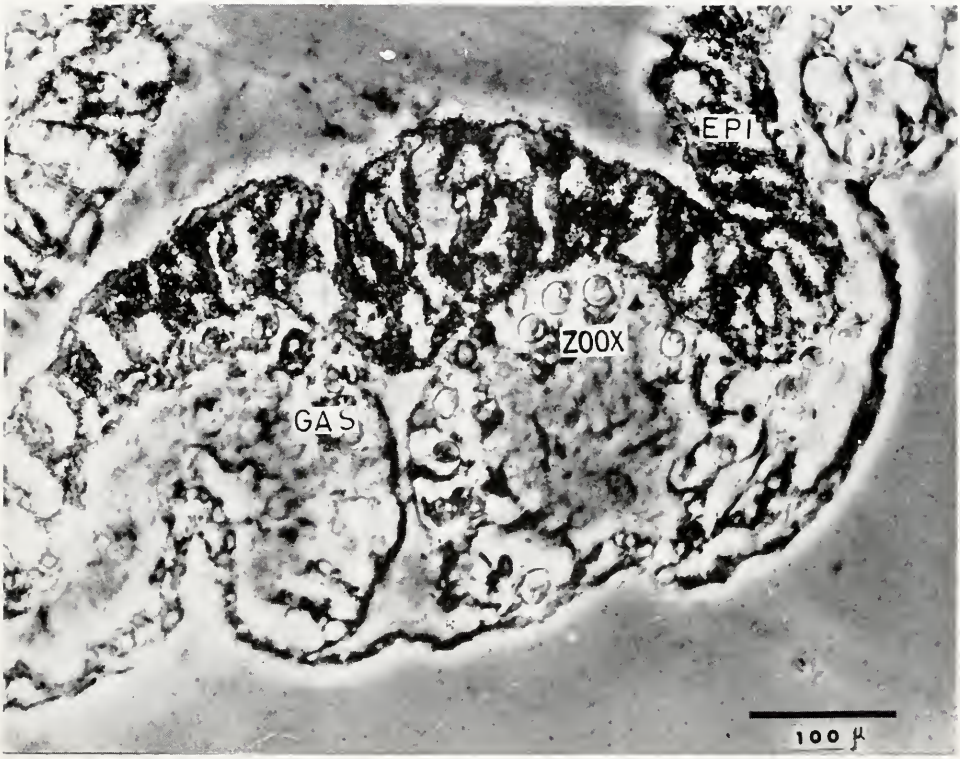


FIGURE 4. Autoradiogram of *Fungia scutaria* exposed to ^{3}H leucine in sunlight for 1 hour, then washed in running sea water. The radioactivity is restricted to the epidermal cells as shown by the distribution of the dark silver grains in the overlying emulsion. Activity first appeared in the tall epidermal cells, then spread over the cellular tissue except for the zooxanthellae, mesoglea and mucus glands. Compare this with figures 6 and 7 for alkaline phosphatase and P.A.S. The dark control shows the same pattern of activity; phase contrast.

from extremely dilute solution. There is also some preliminary evidence that certain Gorgonacea and Zoanthidea have similar abilities.

In the stony corals, the absorption of dissolved organic matter takes place mainly in the epidermis of the column wall, tentacles, oral disc and stomodaeum, *i.e.*, the entire surface in direct contact with the external medium. Autoradiography of the reef corals exposed to very low concentrations of tritiated DL leucine in sea water for one hour and fixed at varying times after labelling show that the activity is initially fixed in the tall columnar cells of the epidermis, whereas much less is present in the gastrodermis, very little in the mesogloea and none in the zooxanthellae (Fig. 4). Twenty-four hours after labelling the activity is more uniformly spread throughout the cellular tissues, except for the zooxanthellae. Epidermal uptake of amino acids by corals is independent of light intensity and absorption occurs even when the leucine concentration is below the threshold of chemotactic response of the test species, *Fungia scutaria*, about $3 : 10^{-9}$ M. Absorption of glucose has since been observed by Stephens (1962).

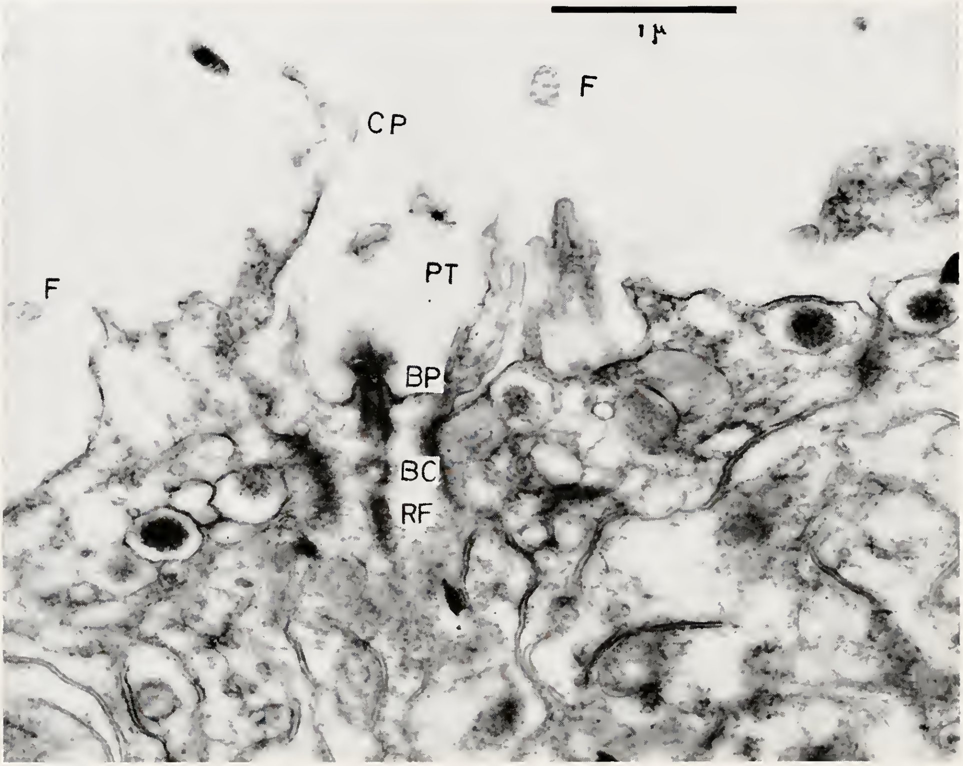
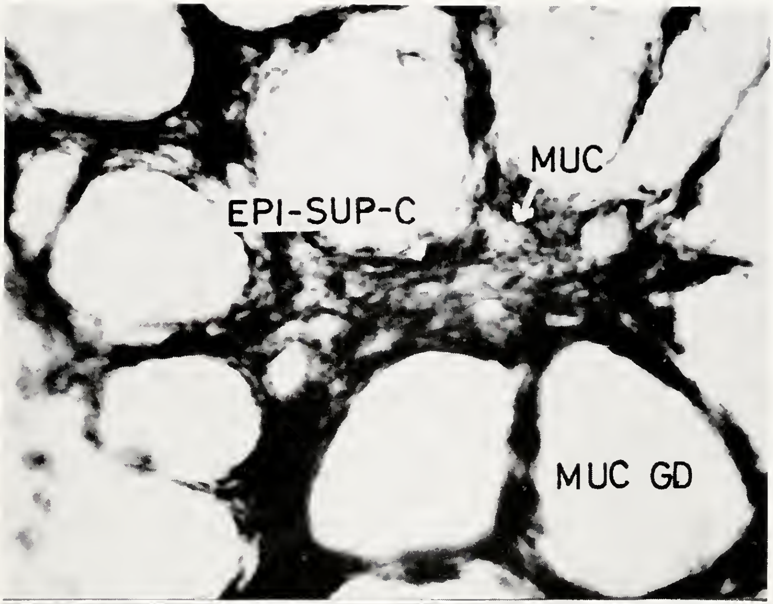


FIGURE 5. Electronmicrograph of the epidermal border of *Astrangia danae* tangentially cut. The epidermis consists of tall columnar cells with a finely granular cytoplasm the free surface of which bears a single flagellum (f) set into a shallow pit (pt) bordered by a circlet of nine to twelve microvilli. The microvilli are especially conspicuous on the collar processes (cp). At the base of the flagellum are shown the basal plate (b.p), basal corpuscle (b.c.) and rootlet fibre (r.f.). The latter has a periodicity of about 670 Å. The arrangement of the microvilli suggests they are modified collars reminiscent of those of choanocytes. The surface membrane shows numerous and very variable cytoplasmic extensions and invaginations suggestive of micropinacytosis. The cell membranes are continuous and show no protoplasmic bridging or syncytial structures.

Electronmicroscopy provides some of the most persuasive evidence that reef corals possess the necessary structural organization for transport of dissolved organic matter across the epidermal barrier. In all species so far examined the epidermis is shown to consist of tall columnar cells the free surface of which bears a single flagellum set into a shallow pit bordered by a circlet of nine to twelve microvilli about 2 μ long and 100 μ in diameter (Goreau and Philpot, 1956). The arrangement of the microvilli (Fig. 5) suggests they are a modified collar reminiscent of that of choanocytes. The surface membrane shows numerous and very variable cytoplasmic extensions and invaginations suggestive of micropinacytosis. Just beneath the surface are mitochondria and endoplasmic reticulum. Thus, not only are the epidermal cells shown to have a surface area many times greater than purely geometric estimates based on light microscopy would indicate, but their ultrastructure is suggestive of a very dynamic cell boundary across



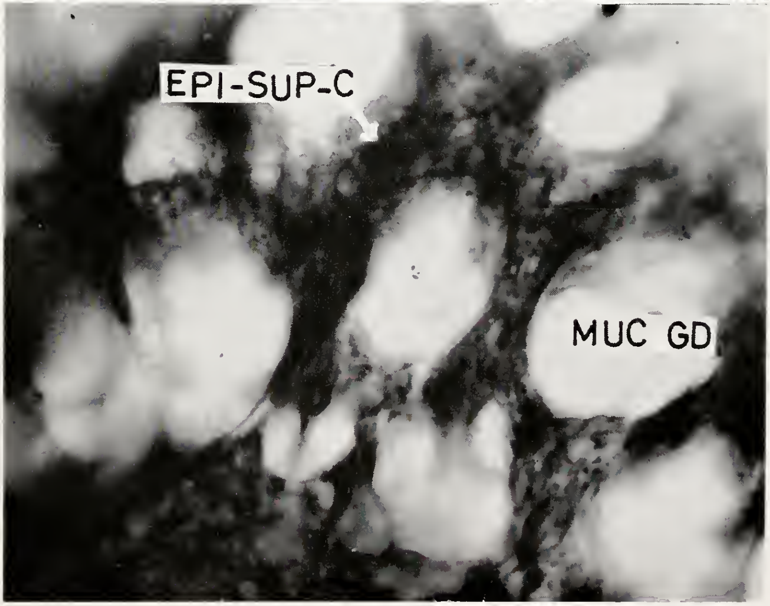


FIGURE 6. Tangential section through the epidermis of *Colpophyllia natans,* stained for alkaline phosphatase and counterstained with eosin Y. The large lacunae represent mature mucus glands (muc gd). The phosphatase activity is confined to the supporting cells (epi sup c) here seen in cross section. Note that the enzyme has a reticular localization in what appear to be cell membranes. The small phosphatase negative vacuoles (muc) may be due to an early stage in the formation of mucus; magnification is × 5000.

FIGURE 7. Tangential section through the epidermis of *Colpophyllia natans* stained with P. A. S. Compare with figure 6 which shows a serial section stained for phosphatases, and note that both this enzyme and the P. A. S. reactive substance have a similar localization confined to the supporting cells (epi sup c). The mucus glands (muc gd) are negative; magnification is × 3600.

which active transport takes place: the data from the amino acid uptake experiments suggest that the net flux is from outside to inside.

Histochemical studies (Goreau, 1956) have shown that highly active nonspecific alkaline phosphomonoesterases are present in the distal parts of the epidermal cells of corals (Fig. 6). The precise function of these phosphatases is not clear: their extremely sharp and high pH maxima with peaks around pH 11.1 suggest that these enzymes do not act *in vivo* as simple hydrolases, but possibly as phosphotransferases supplying energy for metabolic processes occurring in the outer surface of the epidermis. We have not yet been able to establish on the ultrastructural level whether the alkaline phosphomonoesterase is associated with the microvilli. It is of considerable interest, however, that the localization of the enzyme within the coral epidermis is identical with that of a P. A. S. reactive non-metachromatic neutral mucopolysaccharide (Fig. 7). A similar spatial association of alkaline phosphatase and neutral mucopolysaccharide was found by Moog and Wenger (1952) in absorptive and secretory organs of several vertebrate and invertebrate groups. In spite of their phyletic disparity, the epidermal cells of scleractinian corals and the absorptive epithelia of mammalian kidney and duodenum are remarkably similar in general features of their ultrastructure, histochemistry and functions, having in common a large free surface area due to microvilli, high concentrations of alkaline phosphomonoesterases associated with neutral mucopolysaccharide at the free cell border, and being capable of active transport of dissolved organic substances against a concentration gradient. In view of these considerations, it is not unlikely that these epithelia also perform similar functions.

Discussion

After many years of controversy, much remains to be learnt about the nutrition of hermatypic corals. It is significant that, in distinction to ahermatypes, they exhibit a wide range in size and form of the polyps. This could well indicate a correspondingly wide range of specialization for dealing with food material extending from living animals to detritus and to particulate or dissolved material of animal origin.

Corals such as *Favia, Euphyllia* or *Mussa* with large polyps can be observed to feed exclusively on animal prey, *e.g.,* small fish and large zooplanktonic organisms or fragments of flesh (never vegetable matter), in precisely the same manner as do ahermatypic corals such as *Tubastrea* or *Balanophyllia* and all Actiniaria. But, to the extent that these may be available, they may also absorb dissolved or colloidal matter through the epidermis by the mechanisms described above. Where polyps are smaller but still possess adequate tentacles, for instance, *Porites* or *Pocillopora,* and where ciliary currents beat toward instead of away from the mouth (Yonge, 1930a), a primary diet of smaller planktonic animals with particulate and/or dissolved organic matter may reasonably be postulated. In the extreme case of the agaricids (which are very common on reefs) such as *Pavona, Psammocora* or *Agaricia* with minute, and in some cases (*e.g., Pachyseris*) nonexistent, tentacles around very small mouths, particulate food must consist almost entirely of fine fragments of organic matter from the smallest zooplanktonic organisms downward.

In such corals ciliary currents would appear to assist the boundary layer water in conveying the finest material across the surface where the stimulus of animal matter in any form (down to amino acids) will cause mouths to open and mesenterial filaments to be extruded through them. These remarkably efficient organs for combined digestion and absorption of animal matter here take over the function of the tentacles. They extend out of the mouth or other openings in the tissue to seize and enwrap food particles which they may digest and absorb outside the coelenteron (Yonge, 1930a; Abe, 1938). In no scleractinian are the filaments reduced as they are in alcyonarians such as *Xenia*.

While it is now abundantly established that material does pass from the zooxanthellae into the tissues of the host coelenterate—actiniarian, zoanthid or scleractinian (Goreau and Goreau, 1960; Muscatine, 1967, 1969; Von Holt and Von Holt, 1968; Trench, 1971a, 1971b, 1971c; Lewis and Smith, 1971)—the precise significance of this, in the context of the nutrition of the animal, still remains to be determined. Certainly the few species of temperate water actinians which harbor zooxanthellae appear in no way more efficient than the majority which do not. In the bivalve Tridacnidae there is an equally well established passage of soluble material into the blood stream from zooxanthellae (which are later digested in phagocytic blood cells) (Yonge, 1936; Goreau, Goreau and Yonge, 1966). This material rapidly becomes incorporated into the byssus, crystalline style, periostracum and mucus indicating its possible use in the synthesis of these secretions in a manner similar to that described in the sacoglossan gastropod, *Tridachia* (Trench, 1969; Trench, Greene and Bystrom, 1969).

Maintained in darkness, some scleractinians can survive the eventual loss of the zooxanthellae, others cannot. This may not necessarily imply that the latter are suffering from starvation, it may equally be a consequence of the change in the internal environment. Normally the zooxanthellae automatically remove the waste products of metabolism, notably CO_2 with sources of sulphur, nitrogen and phosphorus needed for protein synthesis. Not all hermatypic scleratinians may have the capacity for the efficient removal of these. Franzisket (1970) describes how, after exposure to darkness and consequent loss of zooxanthellae, the tissues of *Porites* atrophy but when exposed to light and reinfected with algae regeneration rapidly occurred. But it remains to be determined whether atrophy and subsequent recovery were the consequences of removal and then restoration of food supplied by the zooxanthellae or of inadequate metabolism when deprived of the algae which normally remove waste products. The major problems ahead involve adequate evaluation of the precise energy needs of corals and the nature of available supplies—zooplankton and particulate or dissolved organic matter of animal origin—in coral reef seas. The spectacular success as reef builders of the hermatypic Scleractinia has tended to obscure the very small amount of living tissue actually present and so exaggerate the amount of food required.

Apart from the discussion, this paper was in rough draft at the time of Professor Thomas F. Goreau's death. Nora Goreau and Maurice Yonge wish to express their gratitude to Willard Hartman, David Barnes, Judith Lang and Robert Trench for criticism of the manuscript. We acknowledge with thanks the assistance of Peter Hunt who photographed our autoradiogram for Figure 4 and further photographic help from Thomas J. Goreau and E. A. Graham. This work was in part supported by the National Science Foundation, the U. S. Office of Naval Research and the Public Health Service of the U. S. Department of Health, Education and Welfare.

SUMMARY

The assumption that reef corals are wholly autotrophic due to the presence of zooxanthellae is questioned. Reef corals lack the behavioral and structural specializations for an autotrophic existence comparable to that found in the xeniid octocorals and zoanthideans which appear to depend upon zooxanthellae for their food.

The heterotrophic nutritional activities of reef corals, as observed both in the field and in the laboratory, include the following: (1) specialized carnivorous feeding, primarily on zooplankton, facilitated by ciliated currents and mucus, direct transfer of prey to the mouth by the tentacles, or extracoelenteric feeding by the mesenterial filaments; (2) unspecialized detritus feeding, involving the use of a wide range of organic matter of animal and perhaps of bacterial origin; (3) direct utilization of dissolved or colloidal organic matter as suggested by the uptake of amino acids by the epidermis and by the ultrastructural, histochemical and physiological features of the free cell border.

Water circulating within the reef, the boundary layer water, is in a continuous and dynamic exchange with the trophic structure of the reef, recycling nutrients with the benthos and making the suspended particulate matter a possible food source for corals.

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ENDOSYMBIOTIC BIOLUMINESCENT BACTERIA FROM THE LIGHT ORGAN OF PONY FISH

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The Leiognathid (pony) fish, which occur in the shallow coastal waters of the tropical and subtropical Indo-Pacific region, are capable of emitting a bright light from their ventral surface. In these small fish, as in several other (but not all) bioluminescent fish, the source of light is symbiotic luminous bacteria, maintained within a special organ (Harvey, 1952, 1958; Buchner, 1965). The evidence that bacteria are involved as symbionts has come from microscopic observations together with the fact that cultures of luminous bacteria have been obtained from the organs (Harms, 1928; Haneda, 1940, 1950). In the present experiments additional proof of the bacterial origin of the light is presented, together with evidence that the symbiotic bacteria are distinct from many of the free living luminescent bacteria which may be isolated directly from sea water in the same area.

In different groups of fish there are very different and sometimes highly elaborate types of organs and modes for display of the bacterial light. In pony fish the system involves several special and unusual elements. The organ itself, which surrounds the esophagus like a donut, and communicates with it *via* paired ducts (Haneda, 1940, 1950) is literally packed with bacteria. Upon dissection it is always found to be emitting light, brightly and continuously, irrespective of the time of day or other environmental factors. The light reaches the outside (ventral) surface via indirect and somewhat sophisticated optics. The gut tract makes a loop into the wall of the swim (air) bladder at the site of the light organ. The organ thus faces directly into the swim bladder, and is provided with an eyelid-like flap which can control the amount of light shining into the air-filled bladder. The swim bladder is internally reflecting, being lined with guanine crystals—the same material which is responsible for the silvery skin of many fish (Denton, 1970, 1971). The ventral portion of the swim bladder is only partially reflective ("half-silvered"), and to it attach specialized translucent lenticular muscle cells. The optical arrangement thus takes the light from a small source and causes it to be evenly diffused over a larger area, namely most of the ventral part of the body.

As shown below, it was possible to estimate the total number of viable bacteria within an organ, and to compare this with the weight of the organ. Such counts indicate that a large percentage, if not all, of the bacteria which are crammed into the ducts of the organ are both viable and bioluminescent.

The isolated symbiotic bacteria were compared with free living bacteria isolated directly from sea water in the same area where the fish were collected. Although the bacteria from the two sources had similar colonial morphology, and were the same in certain other special respects, the two appeared to possess distinctly different types of luciferases, corresponding to types previously known from different strains (Hastings, Weber, Friedland, Eberhard, Mitchell and Gunsalus, 1969), but not previously correlated with symbiotic and free living life styles.

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MATERIALS AND METHODS

Collections were carried out in several locations within Sek Harbor, near Madang, New Guinea, where numerous small coral islands and peninsulae provide varied habitats, all within the protection of the coastal coral reef. Fish were obtained by trawling for 5 to 10 minutes on the bottom at depths of 10 to 30 feet uisng a 20 foot work boat. After removal from the net, fish were placed in a holding tank and returned to the nearby laboratory at Maiwara as quickly as possible (usually within 30 minutes). Survival was not good, but some specimens lived in laboratory aquaria for longer than one week. Fresh specimens were used in all instances for isolation of bacteria. The four species named in the legend to Table I were used in this study.

A liquid medium for the growth of bacteria was prepared by adding 8 g of nutrient broth (Difco) and 3 ml of glycerol to 1 liter of sea water. For a solid medium 15 g of agar (Difco) was added. Cultures were grown at 26° C with both media.

The light measuring equipment employed a photomultiplier tube (1P21) enclosed in a light tight chamber to which the sample could be exposed by a shutter mechanism, the photometer being similar to that described by Mitchell and Hastings (1971). The output was displayed on a meter and recorded on a high speed Watanabe recorder. Measurements of cell density at 660 nm were carried out with a Beckman Model DB spectrophotometer.

In vitro determinations of luciferase were carried out by measurements of bioluminescence in extracts of cells. Culture aliquots were harvested on membrane filters (Millipore Corp.) or by centrifugation at $15{,}000 \times g$ for 20 min. Extraction was accomplished by osmotic lysis in distilled water (Hastings, Riley and Massa, 1965); cell debris was removed by centrifugation. In the assay the reaction was initiated by rapid injection of 1 ml of 5×10^{-5} M catalytically reduced flavin mononucleotide ($FMNH_2$), into a solution containing cell extract (luciferase), oxygen and aldehyde (either decanal or dodecanal) (Hastings and Gibson, 1963; Hastings, Spudich and Malnic, 1963; Hastings, Weber, Friedland, Eberhard, Mitchell and Gunsalus, 1969).

RESULTS

Bacterial counts

Counts of the absolute number of viable bacteria in the luminescent organ of the pony fish provide a way to estimate their mass in the intact organ. In each determination the organ was removed from the fish by dissection with the aid of a stereoscopic microscope, sterilized externally with 70% ethanol, rinsed with sterile water and weighed. The organ was then ground in a sterile glass homogenizer in a small (~ 10 ml) volume of sterile sea water. Aliquots of several dilutions were plated for counts.

The results of measurements made with five specimens (four species; Table I) indicate that there are between 4×10^9 and 1.2×10^{10} viable bacteria per gram wet weight of tissue. Since the packed-cell wet weight count of luminous bacteria is about 5×10^{10} cells/g (Hastings, Riley and Massa, 1965), it may be concluded that between 10 and 25% of the mass of the gland is made up of viable luminescent

TABLE I

Measurement of numbers of endosymbiotic luminous bacteria in pony fish luminous organs

Specimen number*	Weight of fish (g)	Weight of organ (mg)	Viable cells per organ	Viable cells per g organ
1	90	200	2×10^9	1×10^{10}
2	25	40	1.6×10^8	4×10^9
3	9	10	4.5×10^7	4.5×10^9
4	0.1	~ 0.1	1.2×10^6	1.2×10^{10}
5	1.3	0.4	5×10^6	1.2×10^{10}

* Specimen 1, *Leiognathus equulus;* Specimens 2 and 4, *L. splendens;* Specimen 3, *Equilites novaehollandius;* Specimen 5, *Secutor ruconius.*

bacteria. Although there was no easy way to estimate the amounts of glandular tissue in relation to lumen contents, this result is consistent with the hypothesis that all or nearly all of the bacteria which visibly choke the alveoli are viable.

In the platings as carried out, contaminants from outside would be swamped out by the symbionts, so that observations on the colonies give reliable information concerning the inhabitants of the glands. All colonies were luminescent and uniformly so. No difference in colonial morphology or other characters were observed. As previously concluded by Haneda (1950), it thus appears that a single symbiont strain is maintained in the organ in pure culture.

An isolate from *L. equulus,* designated L-4, was retained for comparative studies of the symbiotic and free living bacteria.

Symbionts versus "free living" isolates

No way was found to estimate the growth rate of the bacteria within the organ, but it might be assumed that the cells are maintained in the exponential phase, as in a chemostat, with the excess passing out the ducts communicating with the gut tract. It thus seems possible that "free living" luminous bacteria, defined as those which may be isolated directly from sea water, are simply escapees from the symbiotic condition rather than true saprophytes. Since earlier studies had indicated that at least some of the symbiotic luminous bacteria appear distinct from free living isolates (Meissner, 1926; Harvey, 1952), this question was examined by comparing isolates from the two sources.

Sea water from various locations in Sek Harbor (near where fish were collected) was inoculated on agar plates. After growth for 10 to 20 hours at 25°, bright spots were readily seen on all plates, and bacteria were picked and streaked to obtain single colony isolates. Twenty two such isolates were obtained from Sek Harbor and studied. One of these, designated as SH-1, was used in the comparative studies.

"Autoinduction" of luciferase

In a liquid culture, the growth rates of symbiotic and free living bacteria were the same within error (Fig. 1). The two were also similar with regard to the "autoinduction" of luciferase, which involves a very unusual control of the luciferase gene (Nealson, Platt and Hastings, 1970). After inoculation of cells into a fresh

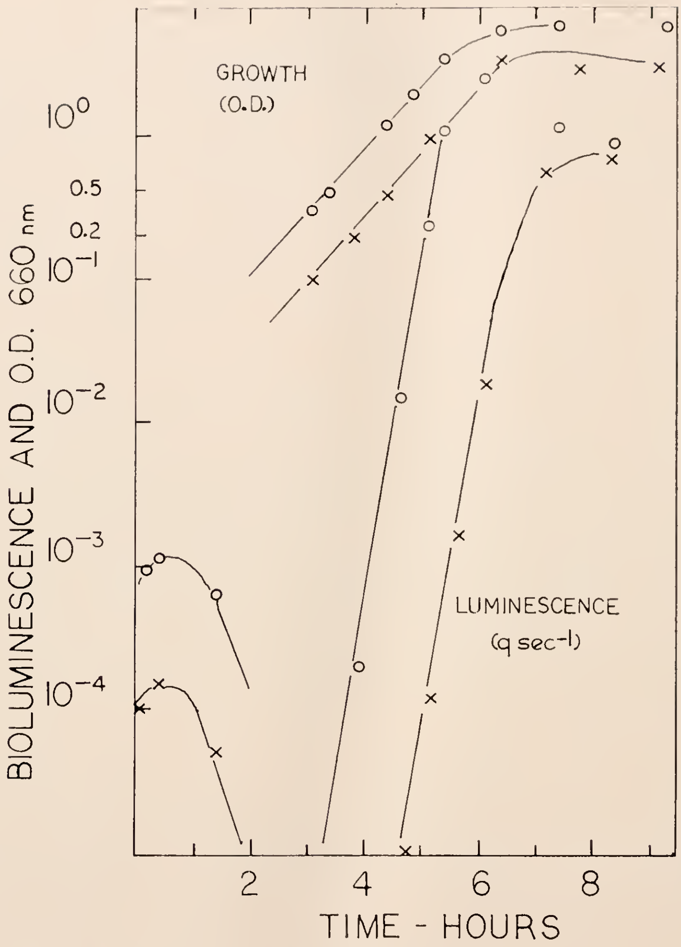

FIGURE 1. This figure shows that growth and luminescence do not occur in concert in free living (SH-1; o——o——o) and symbiotic (L-4; x——x——x) luminescent bacteria. The growth rates and the rates of rise of luminescence are similar for both isolates. The fact that the absolute levels of both cell density and luminescence are lower for the symbiont was not explained. The cultures were isolated from nature two days prior to the beginning of the experiment, and passed through one single colony isolation. Growth was measured by cell density (absorbance) at 660 mm (× 1), using a 1 cm light path; luminescence (× 10^{10}) in q sec^{-1} ml^{-1}. An absorbance of 1 corresponded to about 10^9 cells ml^{-1}.

growth medium, no new luciferase is synthesized for several hours; the actual *in vivo* light emission declines. After this, due to medium "conditioning," a burst of luciferase synthesis occurs, resulting in very rapidly rising bioluminescence.

Occurrence of dark mutants

A second interesting and ill-understood feature of luminous bacteria is their tendency to give rise to "dark" mutants (Keynan and Hastings, 1961). The expression of the gene or operon for luminescence is largely blocked, so that very little luciferase synthesis occurs. Actually, these dark bacteria are not altogether dark; they have an intensity about 10^{-3} of that of the parent, and can (under suitable conditions) revert to the bright form (Keynan, Veeder and Hastings, 1963). Since luminescence consumes energy, this property might have physiological and ecological significance for a form which alternates between two ecologically different habitats. In any event it was of interest and importance to determine whether or not the newly isolated bright bacteria could undergo this "bright-to-dark" mutation. Both the symbiotic and free living strains did. With the isolates used in Figure 1, full grown cultures were allowed to stand without shaking for three days at 30 to 32° C, and then plated for single colonies. Many "dark" forms were derived from both cultures.

It may also be noted that strains isolated from different pony fish organs appeared to differ somewhat in intensity. The reason for this has not been determined, but it is apparently not correlated with the brightness of the intact organ which, by visual estimation, was judged to be quite uniform in the pony fish examined.

Specific luciferases

Although alike in the properties described above, the two isolates (SH-1 and L-4) were found to differ in their specific luciferases. The isolation of two distinctly different luciferases from two culture collection strains has been reported (Hastings, Spudich and Malnic, 1963; Hastings, Weber, Friedland, Eberhard, Mitchell and Gunsalus, 1969). These two luciferases differ in many respects; they may be most conveniently distinguished by the kinetics of their light emission.

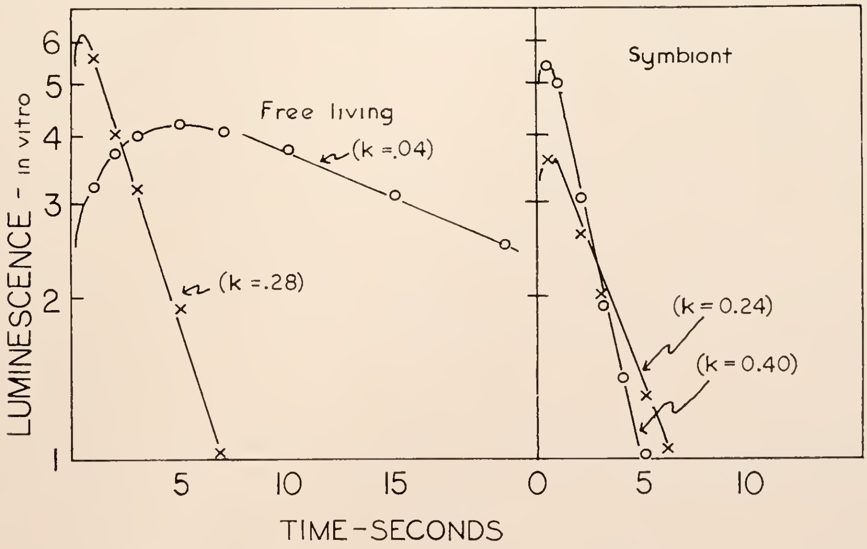

FIGURE 2. Comparison of the kinetics of the *in vitro* bioluminescent reaction catalyzed by luciferases isolated from SH-1 the free living isolate (left panel), with that from L-4, an endosymbiont (right panel). Reaction mixtures were as described in Materials and Methods employing either decanal (x——x——x) or dodecanal (o——o——o); temperature, 26° C. First order rate constants for the decay of luminescence are shown in parentheses.

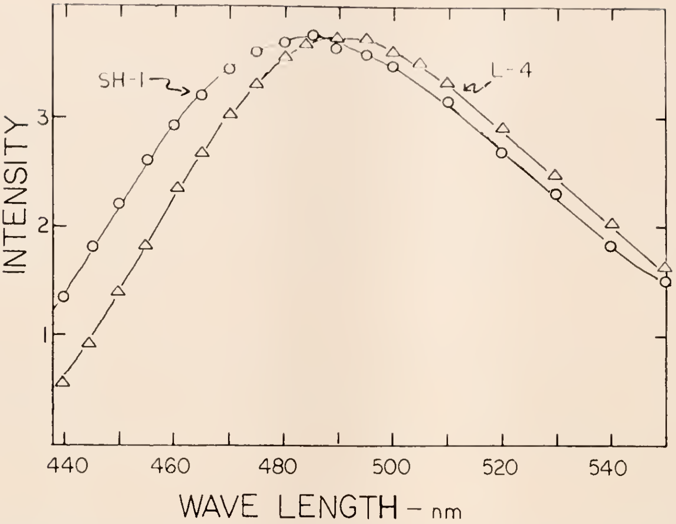

FIGURE 3. Bioluminescent emission spectra for the *in vivo* emission from a symbiont (L-4; Δ) and an isolate from Sek Harbor (SH-1; o).

Using dodecanal, the first order constant (k) for the decay of luminescence was far more rapid for one (strain Pf: *Photobacterium fischeri*, ATCC 7744; $k = 0.4$ sec^{-1}) than for the other (strain MAV; $k = 0.07$ sec^{-1}). On the other hand, using decanal the rate constants were similar for the two luciferases. Luciferases isolated from the symbiotic pony fish bacterium (L-4) and from the "free living" Sek Harbor strain (SH-1) were found to correspond in their kinetics (Fig. 2) to either one or the other of the original two types of Hastings, Weber, Friedland, Eberhard, Mitchell and Gunsalus (1969), not only with dodecanal, but with decanal as well.

In order to determine whether or not any specificity with regard to these luciferases is involved among the bacteria which occur as symbionts, isolates from twenty additional pony fish were obtained, purified, grown and extracted. The luciferase of all was found to be of the fast (Pf) type.

Of the twenty-one additional "free living" isolates from Sek Harbor, fifteen possessed "slow" (MAV) luciferase, similar to SH-1. The remaining six appeared to be similar to the symbionts with regard to their luciferase.

The color of the *in vivo* bioluminescence was also found to be slightly different for the two isolates (Fig. 3). The emission from two of the Sek Harbor strains peaked at about 485 nm, slightly to the blue as compared to the light from two symbionts measured (λ max 491 nm). One of the harbor isolates which had symbiont-like luciferase was tested and found to be similar to the symbionts in the color of *in vivo* emission.

DISCUSSION

From previous evidence, one might always have argued that the bacteria-like particles within the organ are not responsible for the luminescence, and at the same time not viable on artificial media, and that the luminescent bacteria isolated by making a stab from the organ are derived from contaminants. The fact that a major fraction of the weight of the light organ can be attributed to viable bioluminescent bacteria makes it virtually certain that these bacteria are indeed responsible for the light of the fish.

In certain other fish species, notably *Anomalops* and *Photoblepharon,* which possess luminous "headlights" situated externally below the eye, there are bacteria-like inclusions which are believed to be responsible for the luminescence. Attempts to grow these on artificial medium have not been successful, and all contaminants which appear on the plates have been reported to be non-luminescent (Haneda and Tsuji, 1971). Evidence for the bacterial origin of the bioluminescence was provided by demonstrating the presence of bacterial luciferase in extracts.

Knowledge concerning the time and mode of infection of the organ is not available. In the present experiments, luminous bacteria were isolated from the sea water in the vicinity where the fish were collected, and some symbiont types were found. The hypothesis that the endosymbionts occur in a "free living" stage is thus supported. But among the isolates from sea water very many, in fact a majority, of the bacteria appeared to be distinctively different from those cultured in pony fish organs. Therefore, if infection occurs anew in each fish via the gut tract, it would seem that the luminous inoculum would have to be selected from among the several types of bacteria present in sea water. We also conclude that there may be another major group of luminous bacteria which apparently do not infect pony fish. We cannot specify whether these are truly "free living," and saprophytic, or whether they derive from another host with a different specificity. The fact that the bacteria from both groups exhibit in a similar way the special control over luciferase synthesis, and also the interconversion between bright and dim forms, suggests that there is a similarity in the function of the light emission.

Actually, most of the entire spectrum of intriguing questions concerning the biological features of the endosymbiotic relationship remain unanswered. In many respects the system resembles a natural chemostat, but neither its nutritional nor engineering aspects are known. Bacterial growth rates within the organ should be determined, possibly by measuring the rate at which bacteria are exuded. This would probably be easier in certain other fish, where the ducts from the organ lead directly to the outside (Harvey, 1952, 1957).

Finally, it is worth noting that endosymbiosis, which was once treated as something of a biological curiosity, should now be viewed with more interest, especially in modelling the physiology and evolution of intracellular symbionts and cell organelles.

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INDUCED MYOGENIC ACTIVITY IN THE NEUROGENIC HEART OF *LIMULUS POLYPHEMUS*

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Many recent studies have suggested that the classification of neurogenic and myogenic hearts is more ambiguous than was once believed. In general, the hearts of chordates and molluscs were thought to be myogenic while the hearts of arthropods and annelids were thought to be neurogenic (Prosser and Brown, 1961). A notable exception was the heart of the cladoceran, *Daphnia,* which was thought to be myogenic (Baylor, 1942). More recently, myogenic activity has been reported in the heart of other arthropods, notably a moth (*Hyalophora cecropia,* McCann, 1963), an isopod crustacean (*Megaligia erotica,* Ai, 1966) and a cockroach (*Periplaneta americana,* Miller, 1969).

The heart of the mature horseshoe crab, *Limulus polyphemus,* was shown to be neurogenic by the elegant experiments of Carlson (1904a). He demonstrated that if the ganglion was cut, leaving the muscle intact, the heart established different rates of contraction on either side of the cut. If the muscle was cut, even in several places, leaving the ganglion intact, all portions of the muscle beat in synchrony. Upon removal of the ganglion, the heartbeat ceased. However, Carlson (1904b, 1907) later reported that *Limulus* heart apparently had a latent myogenic mechanism that was seen upon immersion of a deganglionated heart in what he described as "isotonic" sodium chloride solution (600 mM). This activity was never seen in normal physiological solution and could be abolished by addition of a small amount of calcium chloride to the isotonic sodium chloride (Carlson, 1904b).

Following Carlson's work, a number of investigators contested his findings of absence of myogenic activity when a deganglionated *Limulus* heart was immersed in physiological solution or sea water (reviewed by Krijgsman, 1952). However, it was Heinbecker (1933, 1936) that first demonstrated convincingly that myogenic activity could be induced in *Limulus* heart under nearly physiological conditions. He reported that a deganglionated heart immersed in sea water and inflated with air or sea water would again begin to beat.

The present report is concerned with a preliminary investigation of the electrophysiological basis for both the sodium chloride-induced and the stretch-induced myogenic activity.

METHODS

Experiments were conducted during summer, 1969 at the Marine Biological Laboratory, Woods Hole, Massachusetts. Large males and females, measuring 15–25 cm across the carapace, were kept in running sea water until used. Hearts were isolated as previously described (Abbott, Lang and Parnas 1969a), pinned out in a wax-filled lucite chamber, and their cardiac ganglia carefully removed. Subsequent preparation differed for the two types of myogenicity. For experiments on sodium chloride myogenicity, hearts were pinned out either with their myocardium intact or after making a ventral midline incision. There was no apparent difference in activity by these two methods and the latter method was preferred since the heart could be mounted lumen side up, permitting easier penetration of fibers by microelectrodes. The hearts were then immersed in 600 mM sodium chloride (Carlson 1904b) or in the concentration of sodium chloride found in *Limulus* physiological solution (444 mM, Chao, 1933).

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A different method of preparation was used for inflation-induced myogenicity. A cannula was inserted into the anterior end of a deganglionated heart and securely fastened with silk thread. The heart was immersed in sea water or *Limulus* physiological solution (444 mM NaCl, 37 mM $CaCl_2$, 9 mM KCl, Chao, 1933) and perfused with air from a pump. When spontaneous contractions began, both ends of the heart were tied off and pinned to the bottom of the chamber, keeping the preparation immersed. Usually the heart remained inflated throughout the entire experiment, the ostia being closed by the internal pressure. If air leaked out, the heart always ceased contracting regardless of how long it had been beating myogenically.

Intracellular electrical activity was recorded by KCl-filled microelectrodes (10–15 MΩ) suspended by fine tungsten wire (0.001″) with a small drop of paraffin.

RESULTS

The normal contraction of *Limulus* myocardial muscle results from a tetanic volley of junctional potentials (j.p.'s) which summate and cause a sustained depolarization of the muscle for about 1.0–1.5 sec (Lang, Abbott and Parnas 1967). These potentials (Fig. 1) reach a maximum of 30–35 mv and are never overshooting (resting potentials, 35–45 mv).

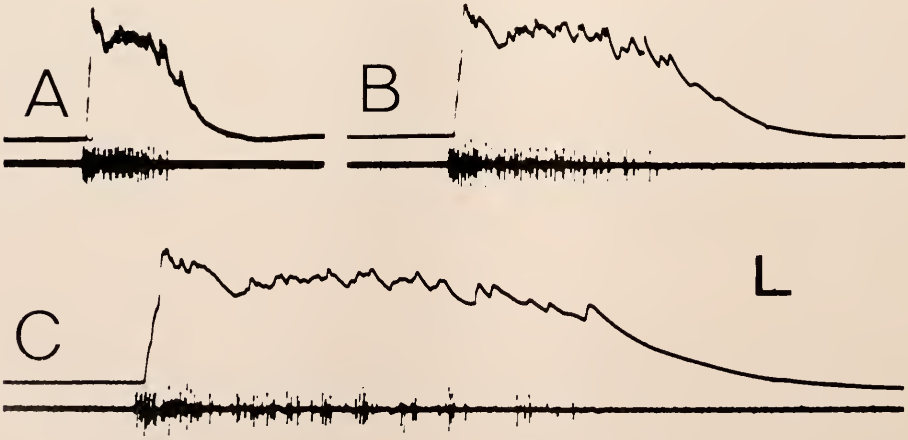

FIGURE 1. Electrical activity of an intact, spontaneously beating *Limulus* heart. Intracellular muscle (upper trace) and extracellular ganglionic (lower trace) recordings show that intracellular activity is due to summation and sustained depolarization from a volley of j.p.'s; calibration: vertical, A–C upper trace, 10 mv; lower trace, 40 μv; horizontal, A, 500 msec, B, 200 msec, C, 100 msec.

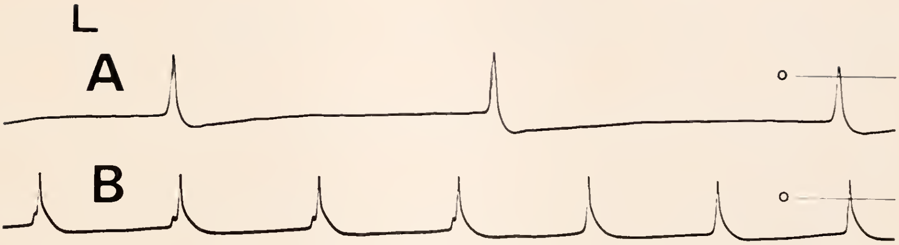

FIGURE 2. Low calcium induced myogenic activity in deganglionated *Limulus* hearts; intracellular activity from two different hearts (A and B) immersed in 0.6 M NaCl. Note pacemaker potentials. Zero potential indicated; calibration: 20 mv, 500 msec.

Sodium chloride myogenicity

When a *Limulus* heart was deganglionated, pinned out, and immersed in 600 mM sodium chloride, rhythmic local contractions began rather suddenly within 10–20 min. This activity lasted longer than 30 minutes but was seldom sufficiently coordinated to result in a synchronous contraction of most or all of the heart.

Impalement of muscle cells in spontaneously contracting segments revealed slowly depolarizing pacemaker potentials which induced rhythmic spiking activity (Fig. 2). Resting membrane potentials were 35–45 mv. Spikes were usually overshooting, and had long durations, never lasting less than 100 msec and often lasting several hundred msec. Often spikes of long duration and small amplitude were observed. Their slow rise time and diminished height suggested that they may have been decrementally conducted from a distant spiking site (see Rulon, Hermsmeyer and Sperelakis, 1971).

The parameters of spike duration and spike height were variable from one recording site to another in a given heart as well as at a given recording site over short periods of time. In addition, two spikes were often recorded sequentially from a single fiber, each having unique parameters of duration and height (Fig. 2B).

Spike height was dependent on external sodium ion concentration. In 444 mM sodium chloride, the same concentration as found in *Limulus* physiological solution, spikes were never overshooting (Lang, 1970; Rulon, Hermsmeyer and Sperelakis, 1970). Decreasing external sodium to 225–250 mM, and replacing with the osmotic equivalent of sucrose, resulted in a decrease in spike height to the point where spikes either became very small or disappeared. Thus spike height was shown to be proportional to external sodium concentration in the range from normal (444 mM) down to 45% of normal, where activity ceased (Rulon, *et al.*, 1970). Further increase in the external sodium concentration resulted in further increase in spike height. In 600 mM sodium chloride, spikes overshoot zero potential by 10–20 mv (Fig. 2).

Addition of double the physiological concentration of potassium (18 mM) to the 600 sodium chloride had no appreciable effect on the electrical or mechanical activity. However, addition of 2–3 mM calcium chloride to the 600 mM sodium chloride eliminated the myogenic activity almost immediately.

Tetrodotoxin (TTX), a potent inhibitor of sodium dependent spikes in excitable tissues, failed to block the myogenic activity when added to the 600 mM sodium chloride even when relatively high concentrations (10^{-5} M) were employed. Likewise, 10^{-4} M procaine, a local anesthetic, had no effect on the myogenic activity.

A characteristic of the sodium chloride myogenicity was the presence of a latent period between immersion of a deganglionated heart in the solution and the time it began to contract. Hearts that were deganglionated and kept in *Limulus* saline for 30 minutes still exhibited the latent period before beginning to contract after immersion in 600 mM sodium chloride. The latent period was also present if an intact, neurogenically beating heart was immersed in 600 mM sodium chloride. The initial effect in this case was an increase in the heart rate and a decrease in the coordination of the beating (Fig. 3B). After five minutes, there was evidence of a regenerative response of the muscle membrane in the form of overshooting spikes on top of the summated j.p.'s (Fig. 3C). After 7 minutes, every other burst from the ganglion had an overshooting spike on top of the neurogenically induced activity (Fig. 3D). These results suggested that a period of synaptic inactivity was not necessary for the myogenic activity but that a certain period of immersion in the calcium chloride solution was necessary, probably to wash out available calcium from the muscle. Whatever the precise cause of the change

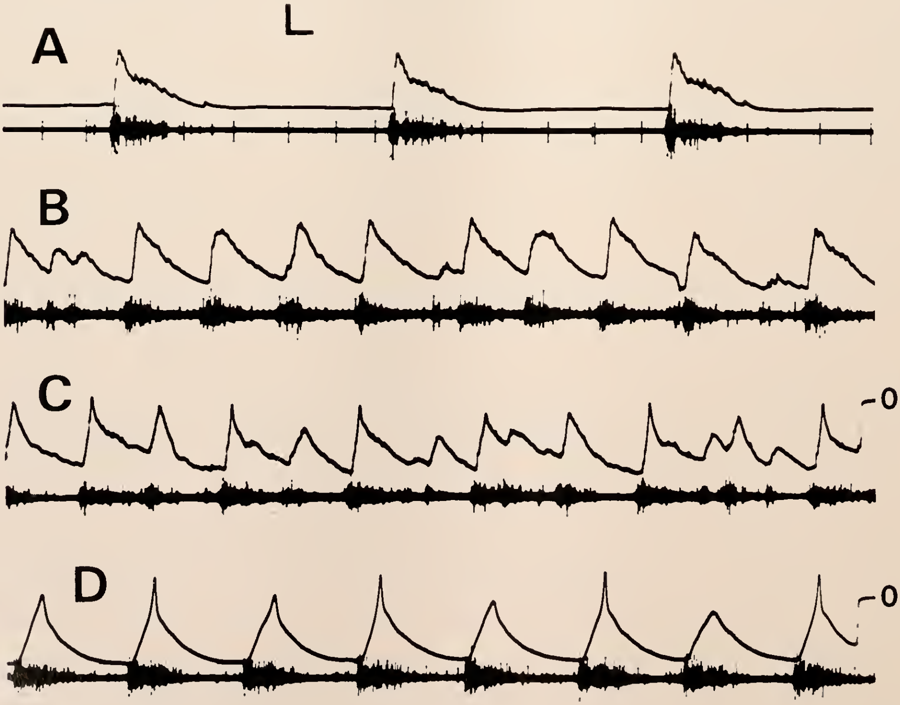

FIGURE 3. Effect of 0.6 M NaCl on spontaneously beating heart. Intracellular (upper trace) and extracellular ganglionic (lower trace) activity; A, control; B, two minutes after immersion in 0.6 M NaCl; C, after second wash in 0.6 M NaCl at 5 min; there appear to be spikes on the peaks of some of the intracellular bursts; D, after third wash at 7 min; every second burst has an overshooting spike; calibration: 10 mv, 500 msec.

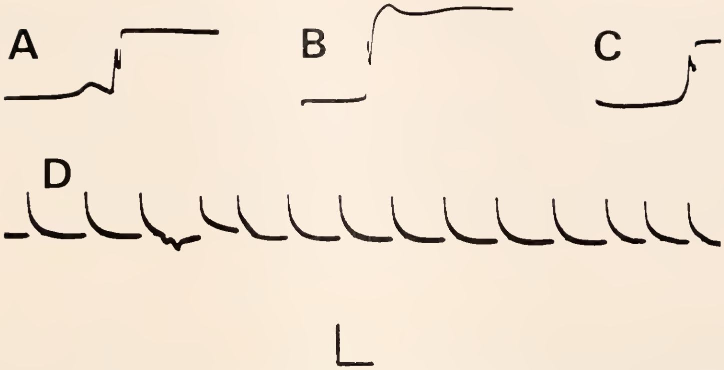

Figure 4. Stretch-induced myogenic activity in a deganglionated *Limulus* heart. Intracellular recording; A–C, the electrode was dislodged due to the mechanical activity, immediately after the peak of the spike; D, train of spikes in a muscle fiber that was not contracting; calibration: 20 mv, 500 msec.

in membrane characteristics, its time course seemed to be reflected in the records of Figure 3C–D. In Figure 3C, spikes are triggered only on the peak of the largest of depolarizations, indicating that they had a high threshold. In addition the fact that not all of the large depolarizations in Figure 3C–D triggered a spike may be evidence of a long refractory period at this stage in development of the myogenic activity.

Stretch-induced myogenicity

When a deganglionated heart was inflated with air and immersed in *Limulus* physiological solution or sea water, it began to contract within 20 min. The activity consisted of a simultaneous contraction of the entire heart circumference, in part of a segment, which occluded the lumen. The contraction wave passed peristaltically in both directions from the point of origin. Normally the contraction began in segment 2, between the first and second pairs of ostia, and was conducted at the rate of 2–4 cm/sec. Activity was observed for over two hours, as long as the heart remained inflated. If the inflation was released, the contractions stopped.

Impalement of the myocardial cells during stretch-induced myogenicity proved to be difficult. In order to induce the contractions, it was necessary to inflate the heart to 2–3 times its normal diastolic diameter. During contraction, the lumen was nearly occluded causing downward movement of the heart wall of about 1.5–2 cm. Consequently, the microelectrode was almost invariably dislodged as the muscle contracted, immediately after a spike (Fig. 4A–C). In a few experiments, impaled fibers exhibited spikes at a rate of 2–3/sec, unaccompanied by contractions (Fig. 4D), thus the microelectrode was not dislodged during this activity.

Membrane resting potentials of inflated hearts did not differ appreciably from those found in a spontaneously beating neurogenic preparation. Stretch-induced spikes were 30–40 mv height and were never overshooting. Spike rise times were 5–10 msec, although spikes with slower rise times were frequently observed.

Pacemaker potentials were absent or very small. Neither TTX (10^{-5} M) nor procaine (10^{-4} M) had any effect on the electrical or mechanical activity of the stretch-induced myogenicity.

Attempts were made to induce myogenicity by stretching a deganglionated heart on a large glass rod or over an inflated balloon in order to eliminate the vigorous concentration. These procedures were unsuccessful even if the hearts were first induced to beat myogenically by inflating with air. It appeared that ability to shorten was necessary for stretch-induced myogenic activity.

Discussion

Myogenic activity has been shown to occur in several arthropod hearts (Baylor, 1942; McCann, 1963; Ai, 1966; Miller, 1969). The presence of a latent myogenic pacemaker mechanism in *Limulus* heart is not surprising when one considers the ontogeny of the species. Carlson and Meek (1908) and Prosser (1942) have shown that the embryonic *Limulus* heart is myogenic from day 21, when the beat is first visible, until day 30 when ganglion cells are first histologically demonstrable. During this period the heart beats peristaltically, the contraction beginning in the anterior end (Lang, unpublished observation) in contrast to the adult heart where the beat begins in one of the posterior segments (Carlson, 1904a).

Sodium chloride myogenicity

Carlson (1904b) observed that a normally quiescent, deganglionated *Limulus* heart would again begin to contract if immersed in isotonic sodium chloride solution. These contractions were shown to be caused by sodium-dependent spikes initiated in the myocardium (Lang, 1970; Rulon *et al.*, 1970). Addition of just a few mM calcium chloride inhibited the contractions (Carlson, 1904b) and the electrical activity (Lang, 1970). The contractions in this myogenic state were seldom coordinated. Segments usually beat independently of adjacent segments. Occasionally two or three adjacent segments beat peristaltically or in near synchrony. It was uncertain whether the activity was conducted electrically or mechanically but it may have been a combination of both. The muscle was sensitive to mechanical stimulation; lightly touching the myocardium caused local contractions. However, decrementally conducted potentials were often observed, so conduction of a regenerative potential throughout the heart muscle was not always complete (*cf.* Rulon *et al.*, 1971). On the other hand, spike potentials probably travel for some distance decrementally, perhaps even from fiber to fiber through the intercalated discs known to occur between them (Jordan, 1917; Lang, 1970; Sperelakis, 1971). If fiber to fiber conduction was present, it probably occurred between longitudinally adjacent fibers (*i.e.* in a circular direction in the heart) since intercalated discs have not been shown to occur between laterally adjacent fibers (*i.e.* in the longitudinal direction of the heart) in *Limulus*. In addition, Parnas and co-workers failed to find electronic coupling between laterally adjacent fibers (Parnas, Abbott and Lang, 1969).

The precise mechanisms involved in the sodium chloride myogenicity are not known. Certainly, the main prerequisite is the absence of calcium ions in the bathing solution. Apparently, concentrations of calcium below 2–3 mM effect the membrane sufficiently to cause periodic transient increases in sodium conductance. This interpretation is consistent with the results of others who also found that calcium effects stability of the membrane of excitable tissue. Low calcium levels were shown to cause repetitive firing in frog (Brink, Bronk and Larrabee, 1946) lobster (Adelman and Adams, 1959) and spider nerves (Rathmayer, 1965). In addition, calcium was shown to affect permeability to sodium ions in squid giant axon (Frankenhaeuser and Hodgkin, 1957; Guttman and Barnhill, 1970) and to have a direct effect on membrane resistance in lobster muscle fibers (Werman and Grundfest, 1961). More specifically, calcium and sodium were shown to compete for the same sites in the membrane of rat (Gage and Quastel, 1966) and frog (Birks, Burstyn and Firth, 1968) neuromuscular junctions and in frog heart (Lüttgau and Niedergerke, 1958). Further work on the present preparation might shed light on whether the ratio of sodium: calcium is important for activity, whether other monovalent cations can substitute for the sodium, and the importance of permeable monovalent anions in sustaining the activity.

Stretch-induced myogenicity

Myogenic activity of adult *Limulus* heart in physiological solution was first convincingly demonstrated by Heinbecker (1933). This activity resulted after internal pressure was increased. The resulting contractions traveled peristaltically, starting in one of the anterior segments, as in the beat of the embryonic Limulus heart. Spread of the contraction was slow (2–4 cm/sec) and the muscle exhibited spiking activity. Again, the mode of conduction in the preparation was uncertain. Spike potentials were nearly uniform in size for all preparations, suggesting active conduction of the spike or at least a uniform conductance change throughout the muscle. However spread of activity via mechanical stimulation of the membrane cannot be ruled out since the ability to contract appeared to be a prerequisite for stretch-induced myogenicity.

Initiation and maintenance of this type of activity appears to be dependent on stretch of the muscle. Stretch is known to affect the *Limulus* heart muscle, causing increased contraction for a given stimulus in a deganglionated heart (Abbott, Lang, Parnas, Parmley and Sonnenblick 1969b). It was uncertain whether this effect of stretch was on the muscle membrane or on the contractile apparatus directly but in light of the present results, it seems likely that stretch can have an effect directly on the myocardial membrane.

It might be suggested that both the sodium chloride myogenicity and the stretch-induced myogenicity had their primary effect by exciting the motor nerves of the heart, which in turn excited the muscle. This appears unlikely since TTX, which was shown to block the motor axons in *Limulus* heart (Abbott *et al.* 1969b) did not affect either type of myogenic activity. But this in itself is somewhat surprising since the spikes, at least in the sodium chloride-induced myogenicity, were shown to be dependent on external sodium concentration. However, another recent report has shown that TTX is not always effective in blocking sodium dependent spikes (Redfern, Lundh and Thesleff 1970). In this regard, it would be of interest to know whether TTX can block the low calcium-induced spiking in other excitable tissues and whether it can block the myogenic beat of the embryonic *Limulus* heart.

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SUMMARY

Myogenic activity could be initiated in a deganglionated *Limulus* heart in two different ways. If immersed in "isotonic" sodium chloride (600 mM) the heart began to contract locally in 5–10 minutes. These contractions were seldom coordinated and were due to overshooting spikes of long duration (100 msec.). Spike height was a function of the external sodium ion concentration. The activity was completely abolished upon addition of 2–3 mM $CaCl_2$.

A second type of myogenic activity could be initiated by inflating a deganglionated heart with air and immersing it in sea water or physiological solution. The contractions began in 10–20 min in one of the anterior segments and travelled peristaltically in both directions. Activity was caused by non-overshooting spikes.

Both types of myogenic activity were resistant to tetrodotoxin (TTX, 10^{-5} M) and procaine (10^{-4} M).

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OBSERVATIONS ON PSEUDOCOLONIAL GROWTH IN HYDRA[1]

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The freshwater hydrozoan polyp *Hydra* (Phylum Cnidaria) has been a favorite tool of developmental biologists for years. It possesses the typical two cell layer hydrozoan body plan in a solitary individual, thereby avoiding the complexity of colonial organization and polymorphism. The great majority of hydrozoan polyps, however, are colonial (Hyman, 1940). Therefore, the question arises, why doesn't hydra also express this type of growth pattern (*i.e.* colonial organization)? What is unique about the hydra which enables it to shed its asexual reproductive products (buds), and thereby remain solitary? Are there any conditions under which hydra does not exhibit a typical solitary growth pattern and assumes a colonial or pseudocolonial existence? If so, what are the characteristics of this transformation?

Previously, Schulz and Lesh (1970) reported that following a rise in temperature, the asexual reproductive products of *Hydra viridis* were not always detached. The result was the generation of pseudocolonial monomorphic hydra. This investigation examines parameters influencing the control and expression of the growth pattern in these pseudocolonial animals. These observations are then compared with existing theories of the control of hydroid growth and polarity in an attempt to determine a basis for colonial *vs.* solitary growth form.

Methods and Materials

H. viridis was mass cultured in eight inch fingerbowls according to the methods of Loomis and Lenhoff (1956), except that demineralized water was substituted for tap water. Cultures were maintained at 22 ± 1° C and were fed daily with *Artemia salina* larvae. The culture solution was changed approximately $1\frac{1}{2}$ hours after each feeding.

Surgical procedures

Routine operations were performed with iridectomy scissors on fully extended, but unanesthetized, 24 hour starved animals. *Regeneration experiments* involved severing the animal completely in the specified region. *Wounding experiments*, alternatively, involved only the removal of a divot of tissue from the specified region.

Grafting experiments employed two distinctly pigmented types of *H. viridis:* untreated *H. viridis* and bleached *H. viridis*. Untreated *H. viridis* was green in color due to the presence of an algal symbiont in the gastrodermal cells. Bleached *H. viridis* had the algae removed from their tissues by treating the animals with glycerine (Whitney, 1907). In this investigation, therefore, four kinds of hydra were available for grafts: green pseudocolonial hydra; bleached pseudocolonial hydra; green normal hydra; and bleached normal hydra.

Grafting was performed in culture solution in a petri dish half-filled with paraffin. Hairs were implanted in the paraffin at right angles to the surface. Host

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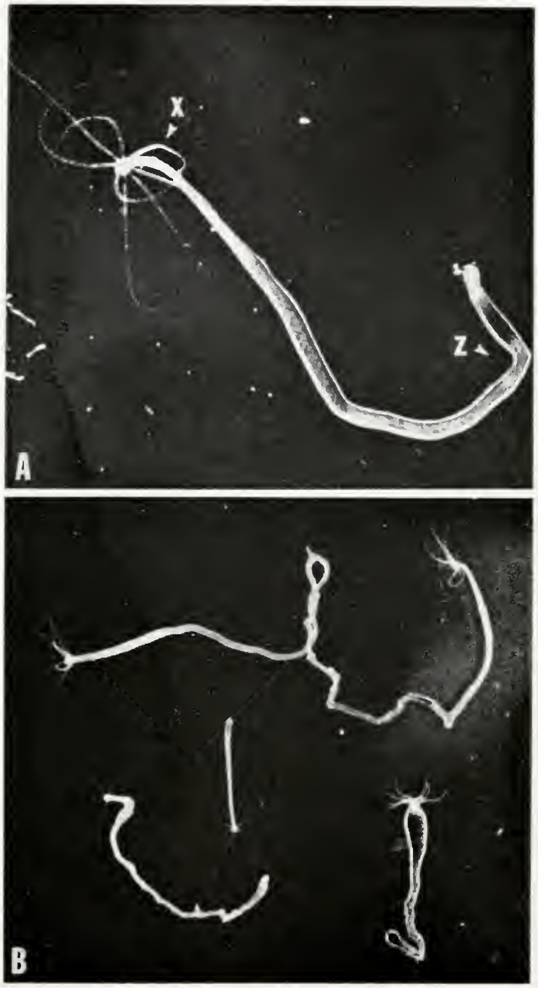


FIGURE 1. Representative monopolar pseudocolonial *H. viridis;* (A.) Single hydra with the regions of wounding indicated by "x" and "z" (see text for description of wounding experiments); (B.) Three monopolar pseudocolonial hydra surrounding a single, relaxed normal *H. viridis,* 4 ×.

animals were impaled on these hairs through the desired graft position. Tissues to be grafted were then also impaled, so that the cut surfaces of the graft and host tissues were in contact with one another. Graft and host parts were held attached to one another with a hair loop. Grafting was completed within 2–3 hours, after which time the hair loop was released, and the organisms removed from the implanted hair.

Histological studies

Animals were fixed for histological studies in Bouins fixative, dehydrated in ethanol, cleared in toluene, and embedded in paraffin. Serial sections were cut at

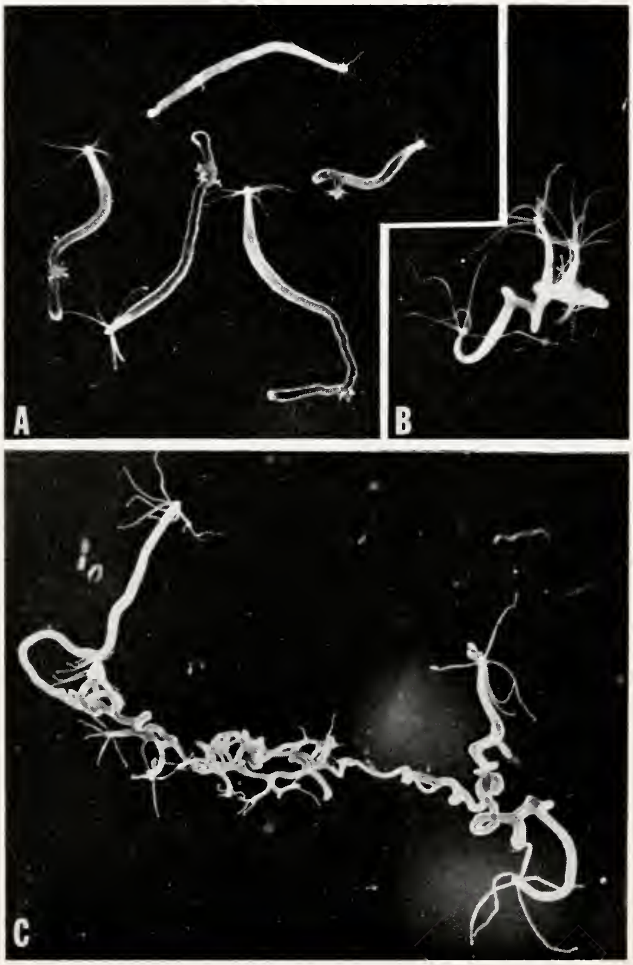


FIGURE 2. Representative pseudocolonial hydra after developing supernumerary hypostomes and/or basal discs; (A.) Initial stages in the development of supernumerary hypostomes and tentacles, 4 ×; (B.) Intermediate development of supernumerary structures, 3 ×; (C.) Typical pseudocolonial form, 2 ×.

5 μ and stained in 0.1% aqueous toluidine blue at pH 8. All histological observations reported were based on sections of a minimum of 10 animals for each experimental situation.

RESULTS

Occurrence of pseudocolonial hydra

A culture of *H. viridis* was inadvertently maintained at 25 ± 2° C for over one month. After this period animals possessing a unique morphology began to appear in the culture (Schulz and Lesh, 1970). These organisms were intensely green in color, and were 2–5 times the length of normal *H. viridis* (Fig. 1). In time these elongated animals also lost the typical apical-basal polarity seen in the hydra. Rather than possessing a single hypostome distally and a basal disc proximally, these hydra developed hypostomes and/or basal discs all along the body column (Fig. 2). Furthermore, the supernumerary hypostomes developed either singly or in clusters. For these reasons these modified hydra have been termed "pseudocolonial" hydra. They will be referred to as such in this report.

Pseudocolonial animals removed from the 25° C culture and returned to normal culturing temperatures (22 ± 1° C) retained their peculiar morphology for as long as one year. (N.B. cultures were not kept longer than one year.) Animals cultured at 20 ± 1° C however, gradually returned to a normal morphology after several months. A constant supply of pseudocolonial forms was maintained during the duration of the investigation by allowing one culture to remain at 25° C. All experiments described in this report, however, were performed at normal culturing temperatures (22 ± 1° C), as detailed in the Methods and Materials section.

Histological organization of pseudocolonial hydra

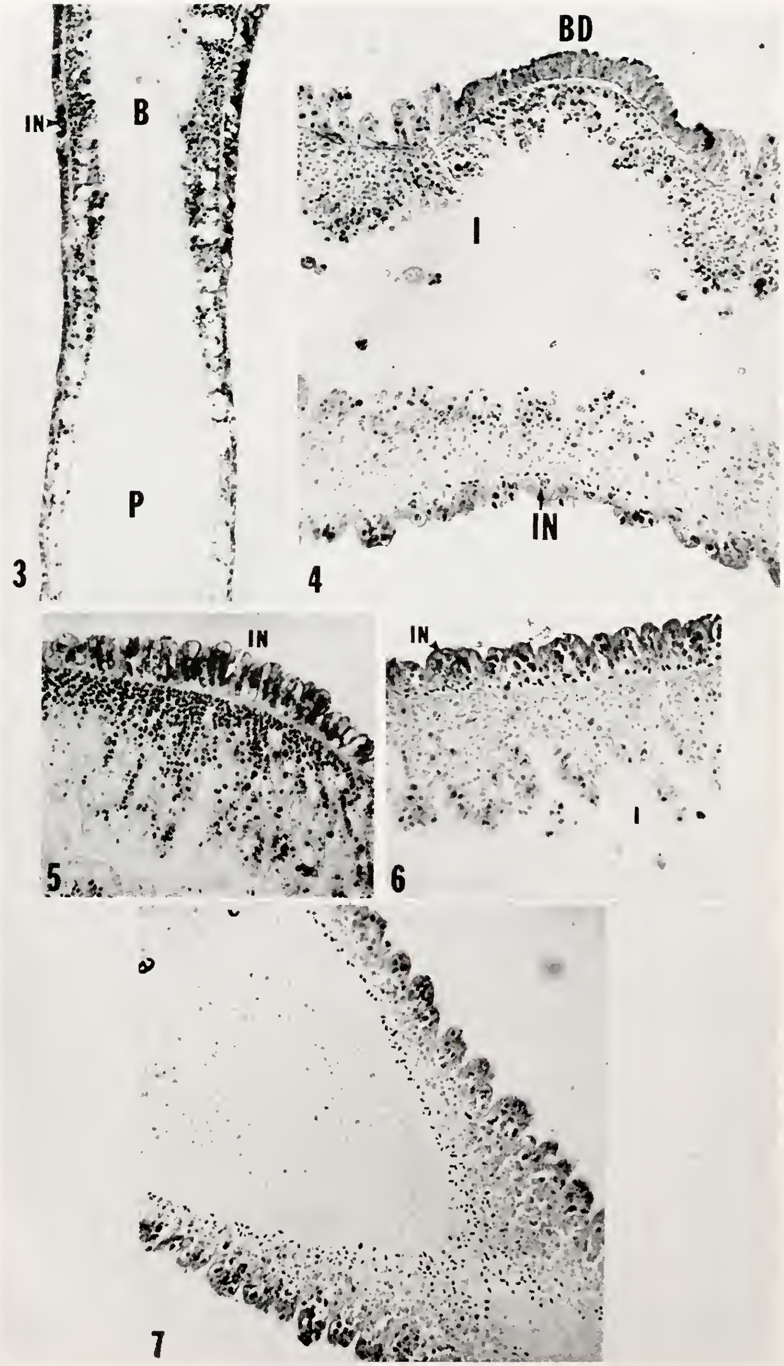
Histological examination of the pseudocolonial hydra revealed marked differences in both the body organization and distribution of cell types from that normally observed in hydra. Normally, proceeding proximally from the hypostome, the body column of the hydra is organized into a "growth" region, gastric region, and budding region. Each of these areas is histologically and histochemically distinct (Burnett, 1959). The budding zone is followed by a peduncle or stalk region, which immediately precedes the proximal basal disc. The peduncle region is characterized by the presence of a vacuolated gastrodermis, almost devoid of both food reserves and symbiotic algae. The epidermis is also characteristically thin and virtually lacks the small basophilic interstitial cells, so prevalent in the regions distal to the peduncle (Fig. 3). Two interstitial cell derivatives, cnidobasts and cnidocytes, are also normally absent from the peduncle. Therefore, the peduncle represents a region of decreased cellular activity. Quite possibly, cells are degenerating here prior to their being sloughed in the region of the basal disc.

In the pseudocolonial hydra, however, there was normally no cytological evidence for the existence of a peduncle. Basal discs developed at varying points along the body columns (see Figs. 2B and C), with no indication of the vacuolation and degeneration characteristic of a peduncle. Interstitial cells and interstitial cell derivatives also persisted in abundance in the areas immediately adjacent to these basal discs. Figure 4 shows numerous isorhizas cnidocytes next to a basal disc. The disc itself appears normal histologically, possessing the typical intense mucus border and elongate epitheliomuscular cells.

Cellular morphology

At the cellular level the only significant changes in the pseudocolonial form were in the number and distribution of interstitial cell derivatives. Interstitial cells *per se* did not appear any more abundant in pseudocolonial individuals. No specific cell counts were made, however, a superficial comparison of Figures 5 and 6 indicates no significant differences would be found. The occurrence of one interstitial cell derivative, however, is markedly increased.

Specifically, increased numbers of isorhizas cnidocytes were noted. This type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Figures 3–7.

of nematocyst is most often concentrated in the hypostome and tentacle regions of hydra (Lesh, 1970). In *H. viridis* they are nearly absent from the body column (Fig. 5). This distribution contrasts markedly with that seen in the column of the pseudocolonial *H. viridis* (Fig. 6). In the modified form virtually a layer of this type of cnidocyte is found just external to the mesoglea. The layered order of these cnidocytes is most apparent in Figure 7 which shows an oblique section through the column of a pseudocolonial hydra.

Growth pattern of pseudocolonial hydra

Ten individual pseudocolonial *H. viridis* were isolated in the "elongate" stage (*i.e.* prior to the development of any branches or supernumerary hypostomes or basal discs) (see Fig. 1). This "elongate" stage has been designated the monopolar pseudocolonial condition, and will subsequently be referred to as such. The growth pattern exhibited by these animals was recorded daily for 5–6 weeks. Accurate positioning of developing supernumerary structures was obtained by diagramming the organisms on graph paper as they appeared on a similar piece of paper affixed to the microscope stage. Two representative patterns are shown in Figure 8A. A representative growth pattern of a typical monomorphic, colonial, hydroid *Cordylophora* is presented also for comparison (Fig. 8B).

These growth patterns readily revealed two differences between the pattern of pseudocolonial *H. viridis* and that of *Cordylophora*. First, the pseudocolonial hydra separated portions of the "colony" at various times during its growth. The *Cordylophora* colony, alternatively, remained intact throughout the 5–6 week observation period. This observation is consistent with Fulton's previous growth pattern studies on *Cordylophora* (Fulton, 1961, 1963). In some instances (see Fig. 8A) the units separated from the hydra pseudocolonies were normal buds (Schulz and Lesh, 1970). Alternatively, the separation occurred at random (*i.e.* unpredictable) positions along the "colony." The latter event resulted in the formation of 2–3 smaller "colonies," often considerably unequal in size.

Superficially, the pattern exhibited by the pseudocolonial hydra recalls both the heterocyte-derived mutant reported by Lenhoff (1965) and the stolonizing mutant of Haynes, Burnett and Deutschman (1964). Both of these mutants often assumed multipolar forms. The occurrence of normal asexual reproduction, however, separates the present growth modification from Lenhoff's non-budding strain. The

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FIGURE 3. Longitudinal section of normal *H. viridis* comparing the budding zone (B) with the peduncle (P). The budding zone (B) possesses an epidermis containing numerous interstitial cells (IN) and gastrodermal cells packed with food reserves and symbiotic algae. Within the peduncle (P), the epidermal layer is thin and contains no interstitial cells. The cells of the gastrodermis are correspondingly thin and vacuolated, 160 ×.

FIGURE 4. Supernumerary basal disc (B) on a pseudocolonial hydra. Isorhizas cnidocytes (I) and interstitial cells (IN) are present in the areas immediately adjacent to the disc. A peduncle region, however, is absent, 160 ×.

FIGURE 5. Gastric region of a normal *H. viridis* showing numerous interstitial cells (IN), but an absence of isorhizas cnidocytes, 160 ×.

FIGURE 6. Gastric region of a pseudocolonial *H. viridis* illustrating a comparable number of interstitial cells (IN) to that observed in Figure 5. The added presence of layer of isorhizas cnidocytes (I), just external to the mesoglea is also evident, 160 ×.

FIGURE 7. Oblique section through a pseudocolonial animal to more directly demonstrate the layered distribution of the isorhizas cnidocytes in this form, 160 ×.

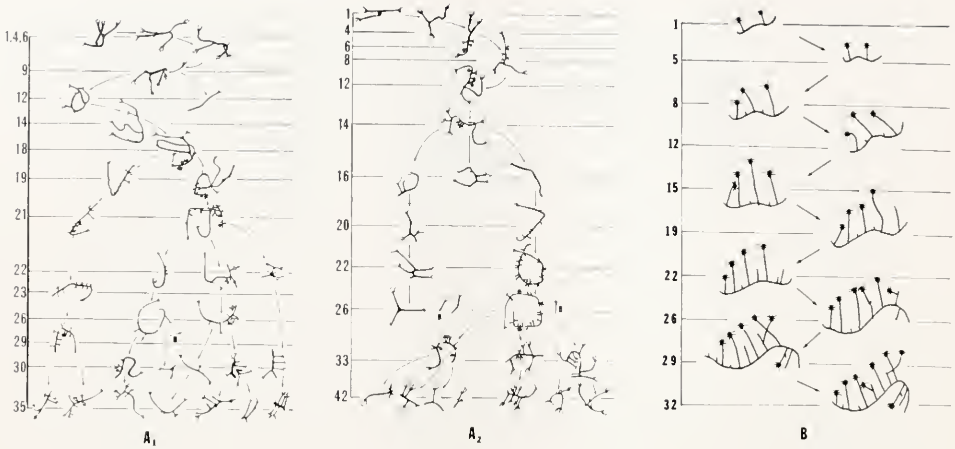


FIGURE 8. Growth patterns of two pseudocolonial hydras (A_1, A_2) and a typical colonial hydroid *Cordylophora* (B). In each of the patterns the ordinate represents days.

further observation that no part of the "colony" lacking a hypostome was ever detached from the parent mass distinguishes this aberrancy from the stolonizing growth modification described by Haynes, Burnett and Deutschman, 1964.

Therefore, although the pseudocolonial hydra was able to retain its asexual reproductive products temporarily, it still maintained the capacity for separation. In this respect it differs from the majority of colonial Cnidarians (Hyman, 1940).

The second difference exhibited in the growth pattern of pseudocolonial organisms was an absence of the spatial regularity in the development of individuals witnessed in the *Cordylophora* colony (see also Fulton, 1961, 1963). This irregularity was most apparent in the formation of hypostomes. Regeneration experiments by many workers have indicated that, with the exception of the peduncle and basal disc, the entire body column of a hydra normally possesses the capacity for hypostome differentiation. Despite this potential, hypostome formation is normally restricted to the distal end of the animal and to the budding region, an area nearly 60% of the length of the body column removed from the existing oral hypostome.

Pseudocolonial hydra developed hypostomes either singly or in clusters at nearly *any* point along the body column. The proximity of another hypostome appeared to have no effect on the subsequent development of additional hypostomal regions. This type of growth is markedly different from that typically exhibited in the hydra. Figure 9 compares hypostome positions along the column in 24 randomly selected, budding pseudocolonial and normal *H. viridis*. Each hydra selected was measured linearly and the position of each hypostome marked on graph paper. The hydra were then divided linearly along the body column into 20 equal segments and the segments containing hypostomes recorded. As all of the hydra possessed hypostomes in segment one, these are not indicated in the figure. In normal *H. viridis* hypostomes were concentrated within sections 12–16 (the budding region). The same analysis performed on pseudocolonial hydra, however, generated the open circle plot in Figure 9. With the exception of section two, hypostomes developed at any point along the body column of a pseudocolonial hydra. In normal *H. viridis*

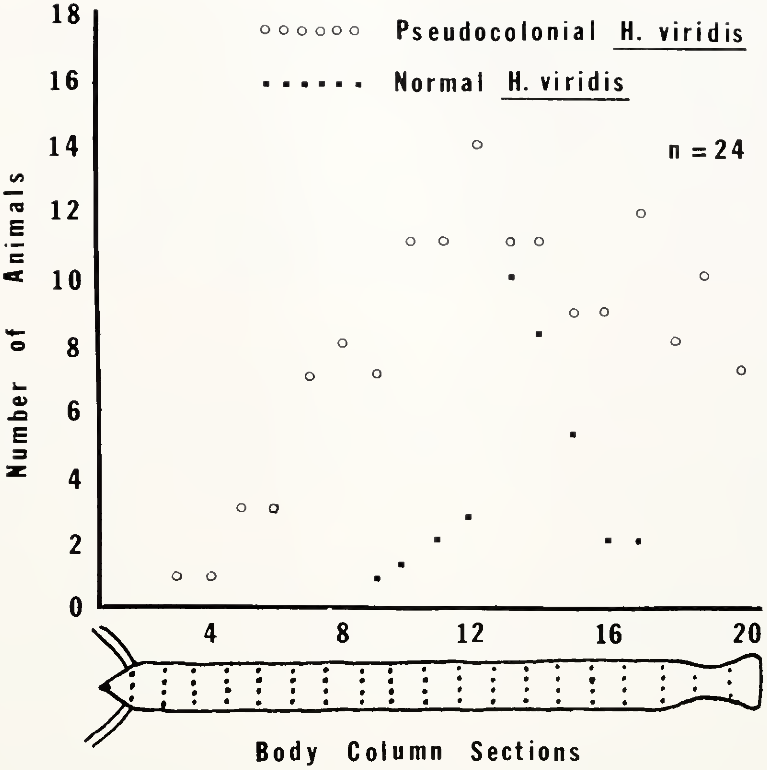


FIGURE 9. Distribution of hypostome formation in normal *H. viridis,* compared with pseudocolonial *H. viridis.*

hypostomes never occurred distal to section nine, nor proximal to section 17. The height of the distributions shown in Figure 9 is irrelevant. Pseudocolonial hydras, by possessing greater numbers of hypostomes, also result in an increased number of animals in each category. The significant point is the distribution of the hypostomes, not the absolute number of these structures formed.

From these observations, therefore, it is also evident that the normal factors operating to control hypostome development in hydra are either not operating or are greatly modified in the pseudocolonial hydra.

Bud development

An additional parameter of the growth pattern of any hydra is the production and detachment of true buds. As both normal and pseudocolonial hydra release buds (Schulz and Lesh, 1970), the similarities and the differences between the processes exhibited in both organisms must be examined.

Buds are normally initiated along the column at a point approximately 60% of the way down the body column. They then move through the budding and peduncular regions, forming a peduncle and basal disc, and are shed in the proximal portion of the adult peduncle (Baird and Burnett, 1967).

In the pseudocolonial hydra buds also initiated at a point removed from a parent hypostome. However, since the parent possessed no peduncle in the vicinity of the budding zone, the bud could not move into that zone and develop its own peduncle in association with similar parental tissue. Buds did, however, progress proximally along the column, form a peduncle and basal disc, and detach. When shed from the parent column the pseudocolonial bud was morphologically indistinguishable from that of a normal *H. viridis.* It possessed the typical distribution of interstitial cells and interstitial cell derivatives characteristically seen in normal animals. Twenty such buds were collected on each of three occasions. All became pseudocolonial within two weeks after being shed.

Basal disc movements

It has been suggested that regions of differential growth may occur in the hydra. Brien and Reniers-Docoen (1949) postulated the existence of a subhypostomal growth region. In this region cell proliferation was presumed to exceed that evidenced elsewhere in the animal. Recently, the autoradiographic evidence of Campbell (1965) has disputed the existence of a growth region or growth center. He has shown that cell divisions occur somewhat uniformly throughout the column of the individual.

One method to test whether any regions of differential growth exist in the pseudocolonial hydra is to mark the column in specific places and to follow the markers as they move along the column. The basal disc acts as a natural marker for such studies. It is not metabolized, is normally incapable of autonomous development, and readily grafts to the body column, providing reliable placement of the marker.

Eleven green pseudocolonial animals were marked with a bleached basal disc grafted in the growth region (or at the top of the gastric region). It made no difference if normal or pseudocolonial basal discs were used as grafts. Five additional animals received two grafted basal discs placed immediately adjacent to one another along the column, also in the growth region. The movement of these grafted basal discs was followed for a three week period. Three representative animals are shown in Figure 10. These diagrams were constructed with graph paper in the manner described in a previous section.

Several points became apparent in examining these movements. First, (1) the basal discs always moved proximally down the column. None moved distally into the hypostome or tentacle region. Also, (2) the total distance moved varied from one animal to another. Third, (3) grafted basal discs moved normally along the column until they reached a point at least the distance of the proximal surface of a normal hydra. At this juncture one of two events occurred. Either a peduncle formed and the pseudocolony split, with the grafted basal disc serving as a basal disc for the newly isolated individual (Figs. 10A and B). Alternatively, if a hypostome developed between the original distal hypostome and the grafted basal disc, no peduncle developed, no separation occurred, and the basal disc continued its movement along the pseudocolony (Figs. 10A and C). The animal shown in Figure 10C was followed for six weeks. At the end of that period the grafted basal disc was still attached to the pseudocolony.

An important point can be made from these observations. In order for separa-

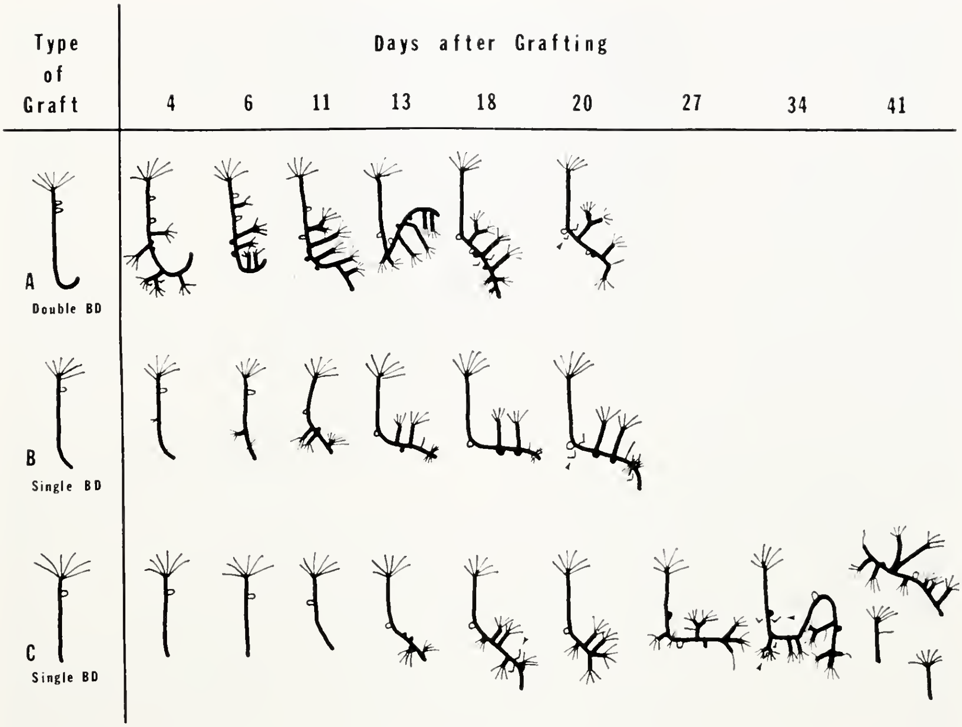


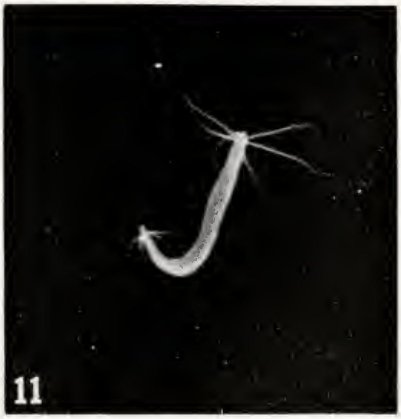
FIGURE 10. Comparison of the movement of grafted basal discs in pseudocolonial hydras. Note that (1) the basal disc always moves proximally down the column; (2) the total distance moved varies from one animal to another; and (3) the grafted basal discs move normally along the column until they reach a point at least the distance of the proximal surface of a normal hydra. Only after this point may separation occur or not occur, depending upon the relative positions of hypostome formation.

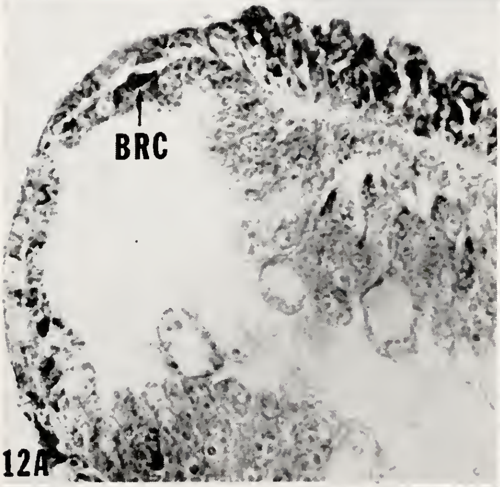
tion to occur in a pseudocolony a peduncle, which is normally not evident in a pseudocolony, and basal disc are required. The basal disc, however, need not be elaborated by the pseudocolony. Any basal disc of the appropriate species may serve as a potential separation point.

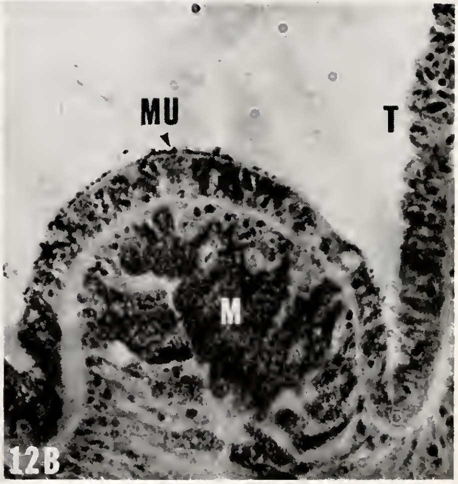
Regenerative capacity

One of the most intriguing aspects of hydra regeneration is that despite the labile capacity for hypostome and basal disc formation within the column, the animal rigorously maintains its disto-proximal polarity during the regeneration process (Burnett, 1961). Colonial hydroids, on the other hand, do not so readily adhere to their existing polarity. Either extremely short or extremely long stolon pieces often will exhibit a bipolar regeneration, forming hydranths at both cut surfaces (Tardent, 1963).

Furthermore, one unique characteristic of Lenhoff's (1965) heterocyte mutant hydra was that upon bisection both cut portions invariably developed hypostomes. Basal discs apparently failed to regenerate in these forms.







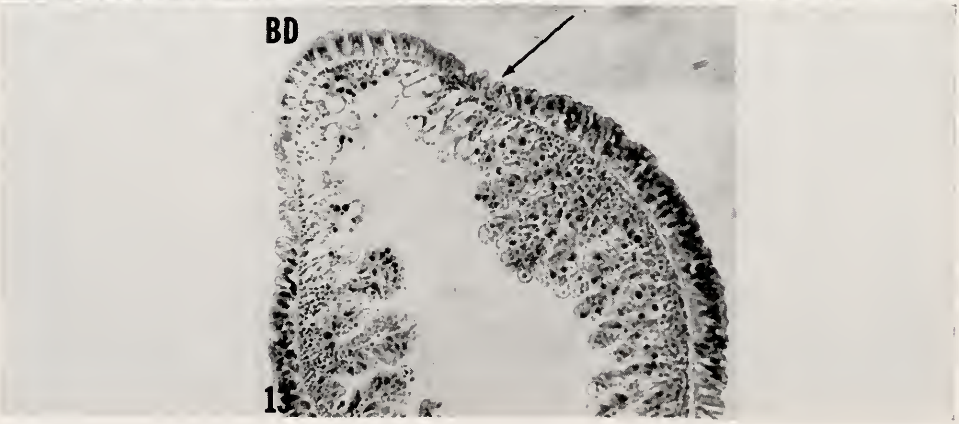


Figure 11. Typical bipolar regenerate which develops if a monopolar pseudocolonial hydra is severed through the lower third of its body column (see Table II for further explanation of the type of cut), 4 ×.

TABLE I

Regeneration following bisection of pseudocolonial and normal H. viridis. *Abbreviations are: Dps = distal half of pseudocolonial* H. viridis, *Dn = distal half of normal* H. viridis, *Pps = proximal half of pseudocolonial* H. viridis, *Pn = proximal half of norma* H. viridis.

Type of cut	Piece	Number of pieces	Structure regenerated at cut surface	
			Hypostome	Basal disc
Bisection of psuedocolonial and normal *H. viridis*.	Dps	15	2	13
	Dn	15	0	15
	Pps	15	14	1
	Pn	15	15	0

Bisection of the pseudocolonial hydra normally resulted in a polarized regeneration (Table I). Occasionally, however, the pseudocolonial animal failed to maintain its existing polarity and developed a bipolarity instead. Of 15 distal halves, 13 regenerated basal discs at the cut surface, corresponding to the existing polarity of the organism. Two, however, developed hypostomes, giving rise to a bipolar regenerate. Likewise 14 of 15 proximal pieces regenerated hypostomes distally. One, however, formed a basal disc.

This result led to the question of what would occur if a pseudocolonial hydra were allowed to achieve its elongated monopolar state (prior to any development of supernumerary hypostomes and/or basal discs) and were then severed through the extended lower third of the animal. This extended region, as shown in Table II, lies below the peduncle and basal disc regions of a normal hydra. Therefore, it is also further displaced from the distal hypostome than any part of a normal animal. When this type of cut was made, the distal pieces regenerated exclusively hypostomes at the cut surface (Fig. 11). Thirty hydra were excised, 30 regenerated hypostomes. This result is directly opposed to the regeneration observed above when the animal was bissected normally; and with the result obtained if one severs a normal *H. viridis* within the lower third of the body column (Table II).

Histological investigations confirmed the results observed grossly (Fig. 12). Within 12 hours after excision of the pseudocolonial hydra very little organized mesoglea was apparent and large numbers of gastrodermal basal reserve cells had collected in the wound area. Few interstitial cells were present (Fig. 12A). By 24 hours, tentacles were forming and increased numbers of interstitial cells were found. Mucous cells lined the gastrodermis and isorhizas cnidocytes were plenti-

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FIGURE 12. Histological sections of regeneration occurring at the cut surface of monopolar pseudocolonial hydra severed through the lower third of the body column; (A.) Twelve hours of regeneration; Note the accumulation of basal reserve cells (BRC) in the cut region; (B.) Twenty-four hours of regeneration. Tentacles (T) have formed and mucous cells (M), characteristic of hypostome formation, line the gastrodermis. The typical external mucous border of the hypostome (MU), is also evident, 160 ×.

FIGURE 13. Histological section of regeneration occurring at the cut surface of normal *H. viridis* severed through the lower third of the body column. After 24 hours regeneration typical basal disc cells (BD) are present, 160 ×.

TABLE II

Regeneration following two types of cuts in the proximal (lower) third of pseudocolonial and normal H. viridis. *Abbreviations are: Dps = distal $\frac{2}{3}$ of pseudocolonial* H. viridis, *Dn = distal $\frac{2}{3}$ of normal* H. viridis, *Pps = piece of tissue isolated from the proximal (lower) third of pseudocolonial* H. viridis, *Pn = piece of tissue isolated from the proximal (lower) third of normal* H. viridis.

Type of cut	Piece	Number of pieces	Structure regenerated at cut surface	
Pseudocolonial and normal *H. viridis* severed in two places. One cut was made $\frac{2}{3}$ of the distance from the hypostome to the basal disc. The second cut was ~3 mm above the basal disc.	Dps	30	Hypostome	
	Dn	27	Basal disc	
			Structure regenerated at cut surface	
			Distal surface	Proximal surface
	Pps	11	Hypostome	Basal disc
		2	Hypostome	Hypostome
		2	Basal disc	Basal disc
	Pn	15	Hypostome	Basal disc

ful. These events are characteristic of hypostome morphogenesis (Burnett, 1959). The mucous border seen in the hypostome region is also present (Fig. 12B).

Normal *H. viridis* severed in a similar manner showed only vacuolated, structureless gastric region cells after 12 hours regeneration. Few interstitial cells, cnidoblasts or food reserves were present in the wound region. By 24 hours typical basal disc cells were found and no interstitial cells were present. In fact, a distinct line (see arrow in Fig. 13) existed below which interstitial cells occurred only rarely (Fig. 13).

Proximal pieces whose origin was entirely within the extended portion of the pseudocolonial animal regenerated diverse structures at their cut surfaces (Table II). Eleven of 15 regenerated a hypostome at the distal cut surface and a basal disc proximally; conforming to the normal polarity of the organism. Two formed hypostomes at each cut end and two formed simultaneous basal discs at the cut surfaces.

These results support earlier conclusions regarding the possible modifications of hypostome control in a pseudocolonial hydra. Obviously, any inhibitory control emanating from the hypostomal region is active only over a portion of the entire body column of the pseudocolonial animal.

Effects of wounding

The possibility of a unique role for the hypostome in the control and expression of form in the pseudocolonial hydra led to the question of what would result if, rather than severing the animal, one only wounded it in a desired region. Two regions were selected for wounding (see Fig. 1A), an area slightly subjacent to the existing distal hypostome (arrow "x" Fig. 1A) and a region in the extended portion (lower third) of the column (arrow "z" Fig. 1A). These regions were selected for several reasons. The area subjacent to the existing hypostome should be influenced by the presence of a hypostome in its proximity. Normal *H. viridis* wounded in this region simply healed the wound and formed no supernumerary structures at the wound site. Nine of 15 pseudocolonial hydra wounded in this region, however, formed basal discs at the wound site. Only 6 healed normally.

A wound in the lower third of the normal animal also was normally healed with no stimulation of regeneration of supernumerary structures. In the pseudocolonial hydra, however, this area is considerably further from the distal hypostome than in a normal animal. Also, the regeneration experiments indicate that this region may not be under any inhibitory influence from the existing distal hypostome. As predicted, 14 of 15 pseudocolonial organisms developed supernumerary hypostomes when wounded in this region.

Role of the hypostome

As evident from the preceding results, a physiological uniqueness of the pseudocolonial hydra appears to be the role of its hypostome in the control of growth and form. Normally, the hypostome is considered the "dominant" part of a hydra from a developmental stand point. It is the source of inductive material that is responsible for the gradient in interstitial cell differentiation along the column (Lesh, 1969, 1970). Furthermore, its presence presumably results in an inhibition in the development of similar structures nearby (Rand, Bovard and Minnich, 1926; Webster and Wolpert, 1966; Webster, 1966).

To examine the role of the hypostome in the control of growth and form in the pseudocolonial hydra, three types of grafting experiments were performed. In all cases grafts were between green and bleached animals so that the graft could be easily distinguished and followed. In the first experiment (1) the hypostome and growth region were exchanged between pseudocolonial and normal *H. viridis.* Second (2), pseudocolonial or normal hypostomes were grafted to the body column of the opposite hydroid type in a typical E. Browne Harvey induction graft (Browne, 1909). Finally (3), the gastric and upper budding regions were excised from pseudocolonial animals and the organisms were then reassembled without the gastric region. Following healing of the graft, a cut was made "x"-days after the graft through the "extended portion" of the animal (*i.e.* the original lower third of the body column). The previous regeneration experiments indicated this extended region normally regenerates a hypostome when severed.

Graft #1: Exchange of hypostome and growth regions. Hypostome and growth regions were exchanged between pseudocolonial and normal hydra to test the capacity of each hypostome to direct its own growth pattern. Ten grafts were performed in each case. In pseudocolonial hydra receiving a normal hypostome (N/PS) no morphological changes from the pseudocolonial form were observed over a 48-day observation period. The animals remained pseudocolonial. Normal hydra receiving a pseudocolonial hypostome (PS/N), however, would not "accept" this hypostome. The hypostome of the first bud that developed invariably became the hypostome of the organism. The pseudocolonial hypostome simply remained attached to the body of the organism and was in time sloughed from the base. These individuals never exhibited a pseudocolonial form nor did any of their buds.

Graft #2: Induction. Normal hypostomes grafted to the body of a pseudocolonial animal (N → PS), (retaining its own hypostome), induced a secondary axis of growth in 16 of 16 grafts. Pseudocolonial hypostomes grafted to the body column of normal hydra (PS → N), however, successfully induced a secondary axis in only 6 of 13 experimental organisms. Furthermore, none of these newly induced outgrowths ever exhibited the pseudocolonial morphology. In the remaining 7 grafts no induction occurred. The grafted pseudocolonial hypostome simply remained affixed to the body of the normal hydra.

Graft #3: Elimination of gastric and upper budding regions. Pseudocolonial hydra that had been reassembled with the gastric and upper budding regions eliminated were naturally smaller than normal pseudocolonial hydra. Consequently the original hypostome was in much closer proximity to the base of the animal. When these animals were severed one day after grafting 23 of 27 animals developed basal discs at the cut surface, while four formed hypostomes. If the graft remained in place for two days before the cut was made, only half of the regenerates (13 of 27) formed basal discs at the amputation site. The remainder developed hypostomes. Allowing the graft to remain longer than two days resulted in still greater proportions of the animals developing hypostomes at the cut surface. The inhibitory effect of the hypostome, therefore, is an immediate one, and appears to be maintained only over a limited growth period. Within 48 hours the situation has changed and hypostome formation is no longer so severely restricted. The animal appears to be reverting to its original pseudocolonial condition in which hypostome formation is inhibited only over a limited portion of the body column.

DISCUSSION

When all of the parameters separating the pseudocolonial hydra from the typical solitary form are considered, the most pronounced and definitive characteristic is a morphological one, the existence of a peduncle region immediately distal to each and every basal disc in the solitary organism. This region, defined by its low metabolic activity, virtual absence of interstitial cells and cnidocytes, and its inability to autonomously regenerate, is also characteristically absent in colonial hydroids. Thus the question arises, is there, overtly, a morphological basis for the occurrence of the pseudocolonial hydra? Does the capacity to form a peduncle possibly separate colonial from solitary cnidarians?

The unique growth effects caused by the presence of a peduncle are most apparent in the growth patterns exhibited by the pseudocolonial hydra. Here hypostomes and basal discs form all along the body column. Separation, however, occurs only if a peduncle develops immediately adjacent to a basal disc. This is most evident in individuals detached from the pseudocolonial form as buds.

A further indication of the role of the peduncle in separation is seen in the experiments using basal discs as markers to detect differential growth along the body column. Here, too, if a peduncle develops, the pseudocolony separates at that point (Fig. 10). If no peduncle forms, no detachment occurs. Interestingly, peduncles form only in those regions where basal discs occur. However, the presence of a basal disc does not always result in peduncle formation.

Of all regions within the hydra, the peduncle has been literally overlooked in both theoretical and experimental considerations of the control of growth and form in the organism. This oversight is not without justification. An isolated peduncle is normally incapable of independent existence. Furthermore, it exerts no apparent inductive growth influence on other regions of the animal (Browne, 1909). Its existence, therefore, has normally been explained as a result of the activity of one of the two presumed organization centers within the animal; the hypostome and/or the basal disc.

Hypostome as an organization center

Clearly, hypostome function is modified in the pseudocolonial hydra. The evidence indicates further that the modification is a suppression or lessening of the rigid hypostomal control normally witnessed in hydra. This conclusion is appropriate whether one is considering a hypostome's inductive (stimulatory) or its inhibitory mechanism of control.

Several observations support the conclusion that pseudocolonial hypostomes possess reduced inductive activity. For example, although the pseudocolonial hypostome is capable of maintaining its own morphological conformation, it neither induces nor remains the dominant hypostome in a normal *H. viridis*. Grafting experiments showed that $< 50\%$ of the pseudocolonial hypostomes were able to induce a secondary axis of growth in normal *H. viridis*. In grafts where a pseudocolonial hypostome was exchanged for the normal hypostome in a normal hydra, the pseudocolonial hypostome graft was not "accepted." The hypostome of the first bud of the normal animal assumed hypostome control of the organism. Normal hypostomes, alternatively, induced their morphological pattern in a pseudocolonial hydra 100% of the time. The latter observation also confirms that the cells of the pseudocolonial form have not lost the ability to respond to inductive materials.

Two additional consequences of hypostome inductive activity in the pseudocolonial hydra that require consideration are (1) the increased number and distribution of isorhizas cnidocytes; and (2) the continuous occurrence of interstitial cells along the body column of the animal. Normally isorhizas are confined to regions of high inductive activity and are thus restricted to the distal areas of *H. viridis* (Lesh, 1970). Interstitial cells are usually prevalent in the gastric and budding regions, but are drastically reduced in number proximal to the budding region, and are virtually absent in the lower third of the body column (*i.e.* peduncle and basal disc regions).

Both of these modified distributions are consistent with the hypothesis that the pseudocolonial hydra possesses a more uniform distribution of inductive influence throughout the body of the organism than one normally finds in hydra. A relatively consistent level of inductive capacity would result in the entire animal possessing nearly maximal concentrations of this activity. The fact that this maximum might be somewhat lower than that found in solitary animals would be irrelevant. In such a situation, events characteristic of high levels of inductive activity would persist. Among these events are the differentiation of isorhizas cnidocytes and a high concentration of interstitial cells (Lesh and Burnett, 1966; Lesh, 1969, 1970). Events associated with decreased levels of inductive activity would not occur. The formation of a peduncle represents one such event (Burnett, 1966).

The presence of hypostomes in virtually all of the 20 segments of a so-divided pseudocolonial hydra attest to a decrease in the inhibitory powers of the pseudocolonial hypostome. Furthermore, regeneration experiments indicate that this inhibition may not simply be reduced, but may also operate over only a limited portion of the body column. The regeneration of hypostomal structures at the proximal cut surface when a monopolar pseudocolonial hydra was severed within the proximal (lower) third of the body column is consistent with this conclusion. Likewise, the formation of supernumerary hypostomes when a pseudocolonial hydra is wounded in the proximal third of the body column also offers support.

The limited nature of the inhibitory effect is further documented by two additional observations. A wound made directly below the distal hypostome results in the development of a supernumerary basal disc, rather than a hypostome. This result is indicative of some degree of inhibition in regions close to an existing hypostome. Also, when the gastric and budding regions are removed from a pseudocolonial hydra, and the hypostome is placed in direct contact with the proximal third of the body column, an inhibition to hypostome formation (as measured by regeneration experiments) is immediately established in this part of the animal. Within 48 hours this inhibition is vastly reduced. The hypostome has now presumably grown further from the most proximal body regions, and thus cannot exert as great an inhibitory effect.

Interpreting these observations regarding hypostomal influences along the body column of the pseudocolonial hydra, the following situations could occur.

(1). The combined effect of a uniform distribution of inductive activities and the innately low level of hypostomal inhibition observed throughout the pseudocolonial hydra may not permit the elaboration of a normal budding zone. Some balance between the factors stimulating (inductive) and those inhibiting hypostome formation must exist to permit bud development. For at present unexplained reasons this balance may not occur in some hydra.

(2.) If no normal bud forms, no region of intense hypostomal inhibition is created along the body column of these hydra.

(3.) In the absence of asexual reproduction, the animal would simply continue to grow. The material and cells normally employed to formulate a bud would not elaborate additional body column. No dilution of interstitial cells to bud formation would occur and these cells would be available throughout the column.

(4.) The combined availability of interstitial cells and the lack of hypostomal inhibition would make the structuring of a peduncle impossible. No separation would occur in such an organism.

(5.) As a consequence of a uniform distribution of inductive substances and the presumed gradient distribution of inhibitory materials, hypostomes could occasionally develop along the column. The presence of a supernumerary hypostome could potentiate two developments. (i) The presence of a secondary hypostome could result in the achievement of the required balance between inhibition and stimulation to generate the formation of a peduncle and basal disc. In these cases separation will occur. (ii) Alternatively, the availability of large numbers of interstitial cells plus the position of the secondary hypostome relative to existing hypostomal structures might result in no alteration of conditions in the body column. Here, the newly formed hypostome would simply remain and grow. In the latter situation one could still expect some manifestations of an increased level of inhibition due to hypostome formation. This increase should be most apparent distal and lateral to the developing hypostome. The inhibitory influence would probably not be in a proximal direction, as here the influence of the existing body column would be the greatest. Encouragingly tentacles form on all secondary hypostomes, and isolated tentacles do occasionally develop in areas near supernumerary hypostomes (see Fig. 8).

The end result of these activities would be the generation of a pseudocolonial organism.

Basal disc as an organization center

Mac Williams and Kafotos (1968) have presented evidence that the basal disc can also act as an inhibitor of its own differentiation. Through grafting experiments they found that the presence of a basal disc, in the vicinity of a proximal wound site, inhibited the regeneration of that structure at the cut surface. Therefore, the possibility exist that the basal disc could control peduncular formation, and consequently, the development of the pseudocolonial condition.

It is quite apparent from the growth patterns and the activity of grafted basal discs that the presence of a basal disc is essential for separation in the pseudocolonial form. The animal, however, has no noticeable difficulty elaborating these structures. They develop singly or in groups all along the body column. Furthermore, the presence of a basal disc does not insure separation will occur.

These facts, together with observations that during normal asexual development a peduncle forms before a basal disc, lead one to conclude that although the presence of a basal disc is important for its own differentiation, it is probably not the controlling agent for a pseudocolonial or colonial existence.

Organization centers in colonial hydroids

Before any ideas about the generation of a pseudocolonial condition can be accepted, however, they must be compared with the growth patterns exhibited in colonial hydroids. Theories exploring mechanisms for the control of growth and form in colonial hydroids have been suggested for many years. Berrill (1961) contains an excellent review of much of the early literature on hydroid polarity. The principal question arising here, however, is this: Could an explanation of hypostomal function, similar to that which has been proposed to explain the occurrence of the pseudocolonial hydra, also at least partially account for the growth pattern seen in *e.g. Cordylophora?*

Initially one must determine what portion of a colonial organism corresponds developmentally to the distal hypostome of a pseudocolonial individual. Two choices present themselves: the hypostome of an individual hydranth or the growing tip of a stolon. Several facts point to the stolon tip as a probable analogue. First, the stolon tip could be considered the "growth center" of a colony. It grows away from the existing colony (Fulton, 1963). Likewise, the hypostome of a pseudocolonial form, by developing an increased column length also displaces itself further from the remainder of the animal. The growth pattern shown in Figure 8 also supports this conjecture, as secondary branches develop first in the proximal region of a pseudocolony and progress distally. This patterning corresponds directly to the growth pattern described for *Cordylophora* (Fulton, 1963).

At the cellular level, the organization and distribution of cell types is also consistent with the hypothesis of analogous roles for the stolon tip and pseudocolonial hypostome. Moving proximally from the pseudocolonial hypostome, one finds a uniform occurrence of interstitial cells and their derived cell types. Only in the buds of pseudocolonial animals is the typical hydra gradient in the distribution of these cells exhibited. The stolon of a colonial hydroid also contains abundant and stable levels of interstitial cells and of their derived cell types. In the uprights and hydranths, however, gradients in cell distribution become evident (Diehl, 1969).

Unfortunately, in a detailed analysis of inductive areas in *Cordylophora lacustris* Moore (1952) was unable to achieve induction with any tissues other than those possessing hydranth differentiation. However, she was able to correlate the capacity for induction with the availability of interstitial cells in the host tissue. Although stolon tips failed to induce secondary outgrowths in her system, considerable technical difficulty was experienced in these grafts. The grafted stolon tip invariably secreted perisarc around itself and separated from the remainder of the tissue mass. This separation could have prevented the necessary tissue interactions and/or temporal requirements requisite to an inductive event. Consequently, the inductive capacity of stolon tips should be reinvestigated.

Therefore, it is postulated that the presence of a level distribution of hypostomal inductive influences throughout the body column of a Cnidarian will ultimately result in the continued availability of interstitial cells throughout the column. Together these facts can prohibit the formation of a peduncle region. This morphological aberrancy restricts the capacity for separation in these organisms and could hence result in the generation of a pseudocolonial or colonial growth pattern.

The ecological reasons why this strain of hydra exhibits a pseudocolonial form when subjected to a condition of environmental stress (increased temperature) remains unknown at this time. Colonial patterns of organization are typically limited to animals of comparatively simple body organization and that reproduce asexually (Barrington, 1967). Conceivably, the possibility to increase the density of individuals in a population for purposes of feeding and sexual reproduction under stress conditions could provide a basis for this modification.

The technical assistance of Mrs. Mary Esther Haggerty is gratefully acknowledged. The author's appreciation is also extended to Mr. Ryland Loos who prepared the final pseudocolonial growth pattern diagrams.

SUMMARY

1. Following a rise in culturing temperatures the reported strain of *H. viridis* grows in length and does not always detach its asexual reproductive products (buds). This aberrancy ultimately leads to the development of a pseudocolonial organization in these animals. Once elaborated, the pseudocolonial condition remains stable at normal culturing temperatures.

2. Analyses of several growth parameters in the pseudocolonial animals reveal that the normal role of the hypostome in the control of growth and form in hydra is modified in pseudocolonial individuals.

3. The principal morphological modification resulting from the altered hypostomal control is the absence of a peduncle region in the pseudocolonial hydra. Normal solitary hydra develop this histologically and histochemically distinct region immediately distal to the basal disc. Colonial hydroids, alternatively, possess no comparable region.

4. Comparing possible mechanisms for the control of growth and form in solitary, pseudocolonial, and colonial hydroids, the observations reported lead to the suggestion that there could be a morphological basis for colonial organization in the Cnidaria (*i.e.* the presence or absence of the capacity for peduncle formation).

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THE IONIC REQUIREMENTS OF TRANSEPITHELIAL POTENTIALS IN *HYDRA*

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The epithelial layers of hydra maintain an electrical potential across the body wall; the enteron is electrically positive with respect to the bathing medium (Josephson and Macklin, 1967). Negative-going action potentials are superimposed on the transepithelial resting potential. These action potentials are called contraction pulses (CP's) because they precede and are probably causally related to contraction of the hydra body column, a shortening due to contraction of muscular elements in epitheliomuscular cells of the ectoderm (Passano and McCullough, 1964; Josephson, 1967). The CP's appear both spontaneously and in response to electrical stimulation. They propagate in the column ectoderm at a velocity of 4 to 5 cm/sec (Kass-Simon and Passano, 1969; Josephson, 1967). There are nerve cells in the ectoderm but their influence on the initiation and propagation of CP's is unknown at present.

The transepithelial potentials of hydra are of interest for several reasons. Hydra live in fresh water. Osmotic experiments and chemical analysis indicate that the cells of hydra are hyperosomotic to the medium and are permeable to water (Lilly, 1955; Steinbach, 1963; Koblick and Yu-Tu, 1967); therefore, there should be a net influx of water. However, hydra have no known organs or organelles for extruding water. It has been suggested that the resting potential of hydra reflects activity of ion transport mechanisms used in osmoregulation (Macklin, 1967). Evidence supporting this is given below. Further, the CP system in hydra may be an example of an epithelial conducting system of which several are known in Cnidaria (Mackie, 1965; Mackie and Passano, 1968). Epithelial conducting systems are likely precursors of nerve cells and an understanding of the physiology of epithelial conduction should give insight into the early evolution of nervous systems. Epithelial conduction has been recently demonstrated in a larval amphibian (Roberts, 1969) suggesting that excitable epithelia may be widespread in the animal kingdom and possibly a significant component in the control of behavior in higher animals as well as in Cnidaria.

In the previous study we used electrical techniques to examine properties of the epithelial layers of hydra (Josephson and Macklin, 1969). The body wall acts as a linear resistance of approximately 5 Kohms-cm^2 to low frequency currents with a density less than about 4 μA/cm^2. With stronger transverse current the column resistance is nonlinear, the nonlinearity having both initial and delayed components. Impedance analysis using A. C. current indicates that the body wall can be represented as an electrical network with a minimum of three time constants. The amplitude and frequency of CP's are unaltered by imposed current, and there is no significant change in column impedance during CP's. These features of the CP are consistent with the hypothesis that the CP-generating membrane forms but a small fraction of the total body wall impedance.

TABLE I

Experimental solutions used. All concentrations are in mM/1.

Solution name	Na^+	K^+	$Tris^+$	Ca^+	Mg^{++}	Cl^-	HCO_3^-	$SO_4^=$	EDTA	Sucrose	$CH_3SO_3^-$
Culture Solution	1.68			1.5		3.0	1.2		0.12		
Normal	1.5			1.5		3.0	1.5				
Na^+free, K^+		1.5		1.5		3.0	1.5				
Na^+free, $Tris^+$			1.5	1.5		3.0		0.6		0.9	
Ca^{++}free, Mg^{++}	1.5				1.5	3.0	1.5				
Ca^{++}free, Sucrose	1.5						1.5			4.5	
Ca^{++}free, $EDTA^-$	1.5								0.48	5.52	
Cl^-free, $CH_3SO_3^-$	1.5			1.5			1.5				3.0
HCO_3^-free, $CH_3SO_3^-$	1.5			1.5		3.0					1.5
$Cl^-+HCO_3^-$free, $SO_4^=$	1.5		1.61	1.5				2.89			
$Cl^-+HCO_3^-$free, $CH_3SO_3^-$	1.5			1.5							4.5

Having characterized the electrical properties of the body wall, we next investigated the role played by each of the ions found in the hydra culture medium. Ham, Fitzgerald and Eakin (1956) found that hydra grew well in a medium containing only $CaCl_2$ and Na_2EDTA. Loomis and Lenhoff (1956) used a medium containing $CaCl_2$, Na_2HCO_3 and Na_2EDTA. In each case additional ions are supplied by the food, which is, in most laboratory cultures, newly hatched *Artemia*. Since the Loomis and Lenhoff medium is most commonly used, the effects of sodium, calcium, chloride, and bicarbonate ions and of osmotic pressure on the generation of the resting potential and CP's were studied and are reported here.

MATERIALS AND METHODS

All of the experiments were performed on *Hydra oligactus* raised as described in Josephson and Macklin (1969). The animals were starved for 24 hours before being used and all experiments were conducted at room temperature (20 to 24° C).

A number of solutions varying in ionic composition was used in our study. The composition of these is listed in Table I. Each of the test solutions was prepared so that it had a calculated osmolarity of 7.5 mosmol, the same osmolarity as the culture solution. The standard for comparison in the experiments was termed "normal solution." This solution differs from the culture solution primarily in the absence of EDTA. EDTA is included in the culture medium to remove heavy metal ions which can be toxic in small concentrations (Loomis and Lenhoff, 1956). Generally the effect of each solution on electrical activity in an animal was obtained (a) when the animal was bathed by the test solution while its gut was perfused with normal solutions, or (b) while the gut was perfused by test solution with the animal bathed in normal solution. The choice of normal solutions as the usual gut perfusate is somewhat arbitrary. The mouth of hydra is normally kept closed so the enteron fluid is separated from and is probably of a composition different than the bathing medium. However, hydra sometimes opens its mouth for extended periods and contact with bathing medium is seemingly not deleterious to the endodermal cells. Also osmotic and ionic gradients across the body wall are minimized by using normal solution as the internal perfusate.

All of the solutions were prepared in distilled water with reagent grade chemicals. When methane sulfonate ($CH_3SO_3^-$) was substituted for the chloride or bicarbonate anions, stock solutions were prepared by titrating methanesulfonic acid with sodium hydroxide to a pH of 7.4 ± 0.2. To obtain the proper pH for the calcium free EDTA solution a mixture of Na_4EDTA and Na_2H_2EDTA was used. When tris base was used, it was titrated with sulfuric acid for pH control.

Other than for these three cases there was no control over pH of the test solutions. However, the pH measured for all solutions used fell between 7.1 and 7.7.

In all experiments a hydra was mounted on the glass holder previously described (Josephson and Macklin, 1969) and shown schematically in Figure 1. The experimental dish holding the external medium had two compartments—a large chamber to permit the animal to be mounted easily on the holder and a small perfusion chamber in which the actual measurements were done. At the start of each experiment the dish was filled with normal solution and the animal was placed on the holder in the large chamber. The holder was then moved horizontally from the large chamber into the perfusion chamber, which had a capacity of 10 ml, and a partition was inserted to isolate the perfusion chamber from the large compartment.

The exchange of fluid in the test chamber was studied by measuring the time constant for the appearance and disappearance of a dye. The dye concentration in the test chamber was monitored by the transmission of light measured with a photocell. The time constant for fluid exchange was about 10 seconds. Assuming good mixing and a time constant of 10 seconds approximately 99.9% of the fluid is exchanged in 70 seconds. To be conservative, external perfusion was continued for 150 seconds each time a solution change was made.

The gut of the test hydra was perfused in these experiments by passing a fine pipet through the holder into the gut. This pipet (P_i in Fig. 1) was connected to a perfusion pump which supplied fluid at a flow rate of 0.7 to 3.5 μl/min. The lower flow rate was only used when flow out of the gut of the animal up into the cup of the holder was retarded due to debris (cells and mucus) collecting in the narrow annulus between the perfusion pipet and the holder. When the flow from the exit was impeded in this way the animals would swell if the inlet flow was not reduced. To maintain a constant pressure head in the gut of the hydra, fluid was removed from the holder cup by a suction pipet, S_i. The gut capacity was estimated to be one microliter from measurements of the outside dimensions of hydra on the test holder. Therefore the turnover of the internal fluid was considerably less than the external fluid. To increase the exchange rate of fluid the internal perfusion tube was placed close to the bottom of the gut so that the perfused fluid rose through the animal as a column. However, because of the slow rate of internal perfusion, only qualitative results for these experiments are reported.

The electrical potential across the body wall was recorded with a high impedance D.C. amplifier and salt bridge electrodes consisting of chlorided silver wires in glass pipets filled with 2 M KCl-Agar.

The transverse impedance of the body wall was determined by measuring the voltage change to imposed sinusoidal current. The current intensity was 0.1 or 0.2 μA and the frequency 1 or 5 Hz. To currents of this amplitude and frequency the body wall acts as a purely resistive element (Josephson and Macklin, 1969). The internal current electrode replaced the internal perfusion pipet so these animals were not internally perfused. After each test with a set of different solutions the impedance of the apparatus alone was determined by removing the animal without disturbing the current and voltage electrodes and again determining the ratio between voltage and imposed current in each of the solutions used. The apparatus impedance varied slightly between the different test solutions because of their different conductivities. The impedance of the apparatus for a given solution was subtracted from that measured with the animal in place to get the impedance of the body column alone.

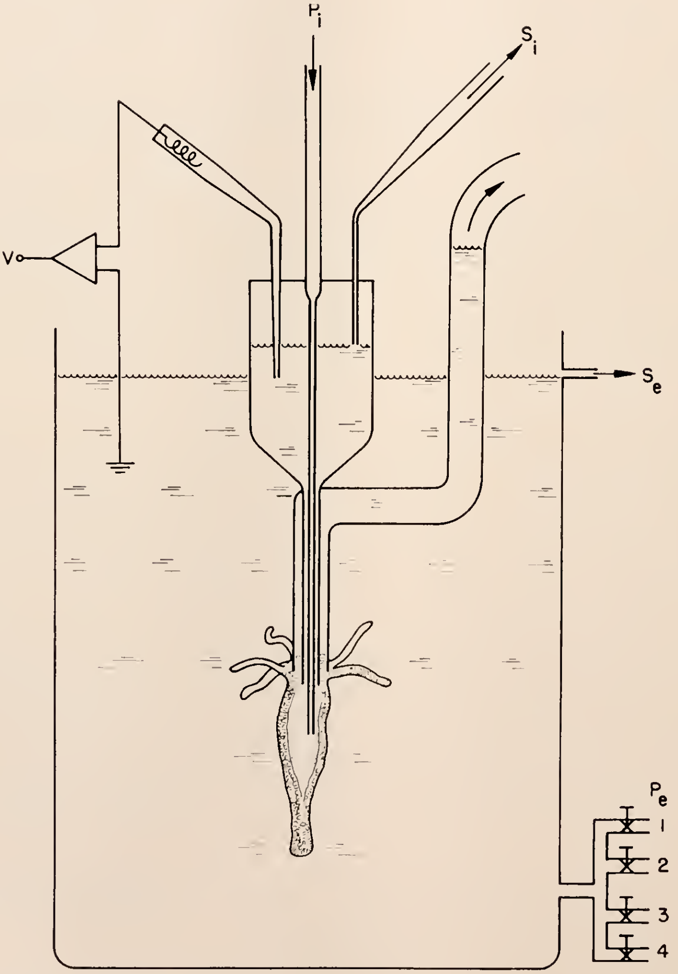

FIGURE 1. The experimental apparatus. An animal is shown on the glass holder held on by suction applied to the unmarked tube. The animal was internally perfused through the pipet P_i and the perfusate was removed *via* S_i. The external fluid was perfused through one of the four inlets, P_e, and removed *via* S_e. The transepithelial potential was measured between the cup of the holder and the bathing solution.

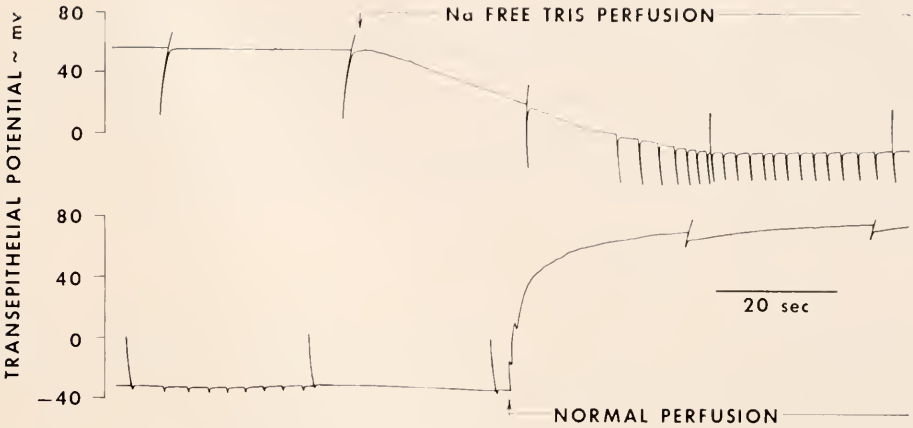

FIGURE 2. Effect of Na-free tris solution on the outside of the animal. The electrical records in this and the following figures were recorded with a curvilinear penwriter. The record begins (top) prior to external perfusion with Na-free tris. The record is broken before perfusion was complete and begins again (bottom) one minutee prior to perfusion with normal solution. Positive going pulses are stimulus artifacts which are separated by 30 sec. Note the decrease in CP magnitude during perfusion and oscillations during the readmission of normal solution. This was the second ten minute period of exposure to Na-free tris for this animal.

When testing different solutions, systematic errors were minimized by changing solutions in random sequences with the use of a random number table. For each solution change several readings were taken. Preliminary tests established that the potentials became stable less than 5 minutes following a solution change. Accordingly 5 to 10 minute test periods were selected for different experimental solutions. For a 5 minute test period, the resting potential was measured at 3, 4 and 5 minutes after the beginning of the solution change. For a 10 minute test period, measurements were made at 6, 7, 8, 9 and 10 minutes. The resting potential for the test period was then taken as the average of the measured values. For any test sequence all test periods were of the same length and the external perfusion during solution changes always lasted 150 seconds. Each test sequence was repeated several times for each animal. The average values of the potential obtained in each test period were themselves averaged. The data reported are a result of the analysis of data from several animals for each separate experiment.

RESULTS

The transepithelial resting potential reported in this paper for the test animals are noticeably higher than the values previously reported (Josephson and Macklin, 1967, 1969). We can attribute this increase to several factors. All previous experiments were done with culture solution (Table I) on the outside of the animal, whereas the standard for comparison in this study was the normal solution (Table I) which did not contain EDTA. A preliminary test indicated that the resting potential is greater when the animal is bathed in normal solution than when it is bathed in culture solution. Second, the perfusion of the gut with normal solution tended to increase the resting potential of internally perfused animals above that of animals which were not so perfused. Third, the resting potential tends to increase for some time after the animal is mounted on the test holder, and so the initial resting potential is somewhat arbitrary. Since the increase was most pronounced for the first fifteen minutes, and since in the previous work we had started the measurement of the resting potential sooner than in these experiments, the increase in reported resting potential is due in part to the experimental protocol followed.

(A). *External ion substitution*

(1). *External cation substitution—sodium.* Sodium was replaced by either potassium or tris ion to determine its effect on the hydra transepithelial resting potential and the contraction pulse. The results with the two substituting ions were similar but not identical. Removing sodium in both cases caused the resting potential to fall. For three animals the average potential in normal solution was 67 mv (range 61 to 75) and in sodium-free potassium solution it was 1.1 mv (range −4.4 to 9.1). Similarily for three other animals the average resting potential in normal solution was 63 mv (range 52 to 77) and in sodium free tris solution it was −19 mv (range −26 to −10). These resting potentials were measured for the last five minutes of ten minute test periods as described above; two to five test sequences were done with each animal. The results show that maintenance of the potential requires sodium in the external medium and neither potassium nor tris will substitute.

The changes in the resting potential at the onset of perfusion with sodium-free solution and at the readmission of solution containing sodium are not symmetrical; the latter is much more abrupt (Figs. 2, 3 and 4). This suggests that the relation

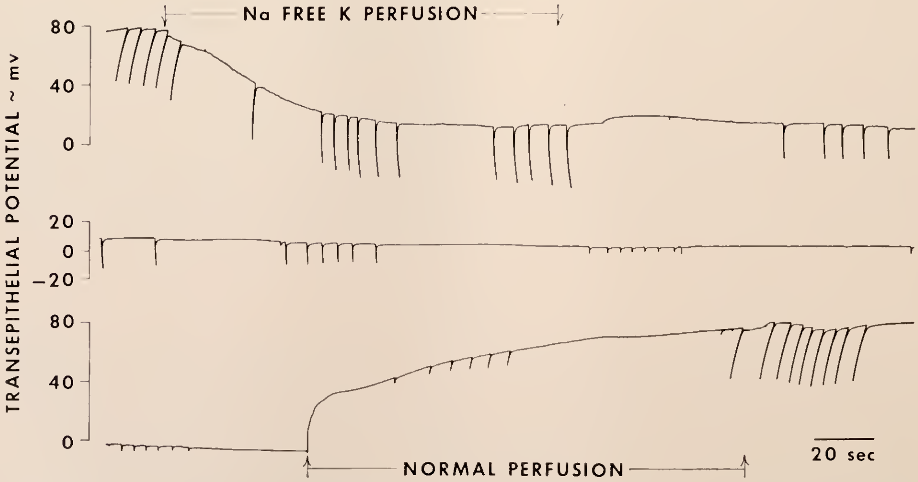

FIGURE 3. The effect of external Na-free-K perfusion. The complete record for the first ten minute period of exposure to Na-free-K for this animal is shown. Note the two step recovery of resting potential following readmission of normal solution.

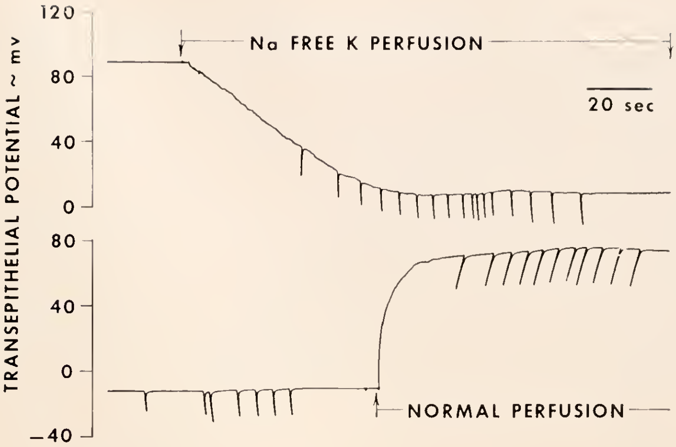

FIGURE 4. The effect of external Na-free-K perfusion. This is a portion of the record for the fourth ten minute exposure to Na-free-K for the same animal as Figure 3. Note constancy of CP size and the one step recovery of the resting potential.

between sodium concentration and the resting potential is nonlinear, and that an appreciable resting potential is present at relatively low sodium concentrations. Since the solution exchange during perfusion is approximately exponential it takes considerable time to reduce the sodium concentration to low levels when perfusing with sodium-free solutions, while the sodium concentration rises steeply when solution containing sodium is readmitted. The resting potential records frequently showed two or three oscillations when sodium was readmitted to the external medium (Fig. 2). We do not know whether these oscillations are inherent properties of the mechanism generating the resting potential or if they are due to sudden surges of sodium rich solution passing the animal in the turbulent inflow, although the former seems more likely.

The CP amplitude often falls during the period when the animal is in a sodium-free external medium (Figs. 2 and 3). The time course of CP decline was examined by stimulating the column electrically to produce CP's at regular intervals. The stimulating electrode was a suction electrode on the basal disc (see Josephson, 1967). The stimuli were 1 to 3 msec current pulses somewhat above CP threshold. Examples of results from these experiments are shown in Figures 2 and 5. The decline of the amplitude of both evoked and spontaneous CP's was most marked during the initial exposures with sodium free solution; in later exposures the CP size showed little or no changes throughout the period in sodium-free solution (Fig. 5 and compare Figs. 3 and 4). The CP generating system seemingly adapts to changes in the external environment. Even when their amplitude has decreased greatly, CP's return to normal size within a few minutes after sodium is reintroduced in the external medium (Figs. 3 and 5).

The resting potential did not show the same type of adaptation during repeated test sequences as did the CP's. However, when the sodium was replaced by potassium, it was noted that if the CP's declined in sodium-free solution then the resting potential return contained two distinct phases when the sodium was readmitted (Fig. 3). An initial rapid phase was followed by slow recovery lasting a minute or more. Only the rapid phase appeared in the later test sequences when there was no CP decline (Fig. 4). When tris was used as a substitute for sodium the potential always showed a rapid rate of return to the control level even if there was a decline in the CP amplitude during the sodium-free period (Fig. 2).

The sodium concentration was varied by decades from its normal value of 1.5 mM to determine the dependence of the resting potential on the external sodium concentration. Concentrations of 0.015 and 0.15 mM sodium were prepared by mixing normal and Na-free-tris solutions. Na_2SO_4 was added to the normal solution to obtain 15 mM sodium. The latter solution had a higher osmotic concentration than the other test solutions and the addition of sulfate both raises the ionic strength and probably decreases the external calcium activity. It will be shown that the transepithelial potential is unaffected by changes in the external osmotic con-

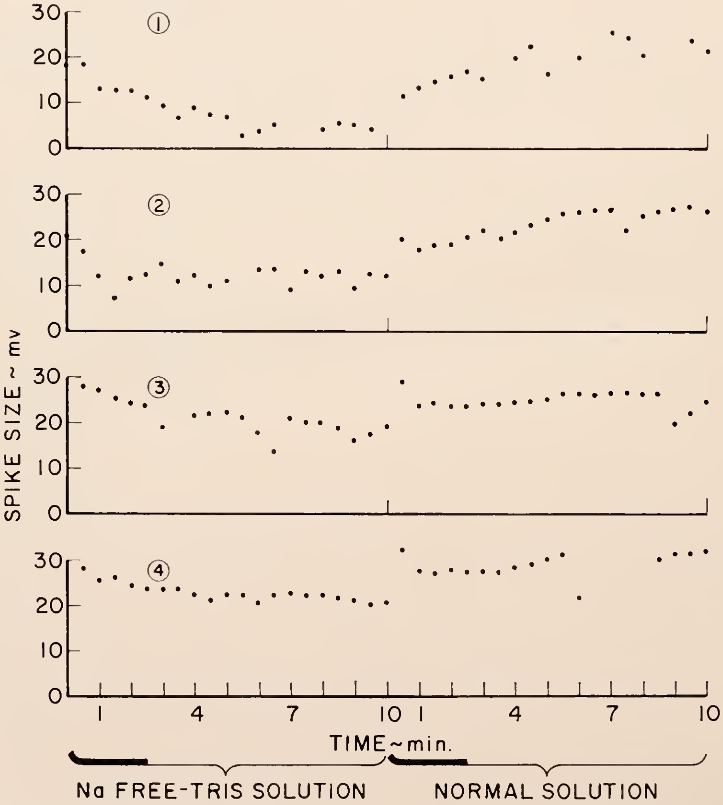

FIGURE 5. Pattern of CP adaptation to external Na-free perfusion. Every 30 seconds a CP was evoked by a stimulus and its magnitude measured. Note the decline and recovery of the CP's in the first fluid exchange sequence and the gradual adaptation with each subsequent exchange. External perfusion occurred during the first 2.5 minutes of each 10 minute period. Occasional missing points are due to increases in the CP threshold. Each time the CP system failed to respond the stimulus intensity was increased slightly until the stimuli were again effective.

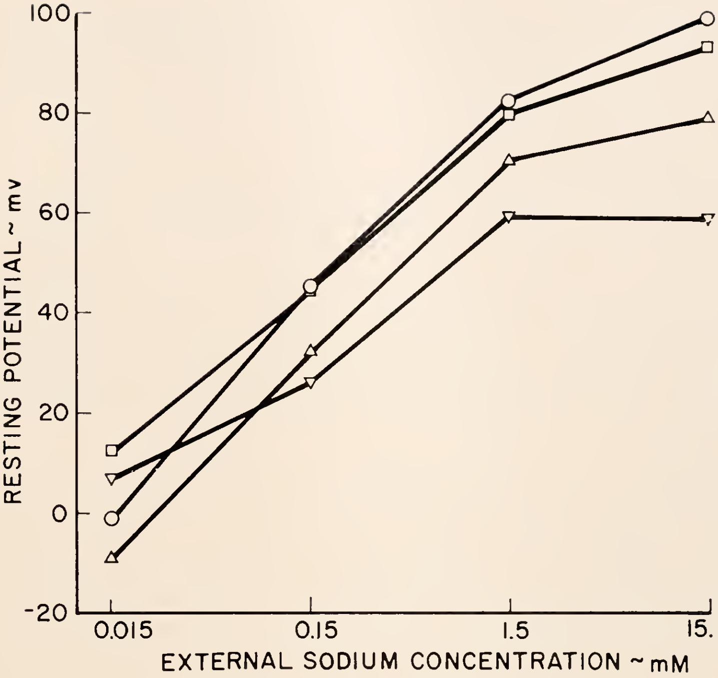

FIGURE 6. Dependence of transepithelial resting potential on external sodium concentration. Records for four animals are shown. Each point is the average of four measurements taken in random order.

centration in this range and that sulfate can replace both Cl^- and HCO_3^- in the bathing solution with no significant change in the transepithelial potential. Although the effects of small changes in external calcium concentration and of ionic strength have not been investigated it seems likely that the changes in transepithelial potential seen with this solution are due solely to the increased sodium concentration.

The resting potential increased monotonically with sodium concentration (Fig. 6). The rise is initially logarithmic but shows evidence of saturating at higher concentrations. The average slope for the initial logarithmic phase (0.015 to 1.5 mM sodium) is 35 mv per decade change in sodium concentration, rather less than the 58 mv per decade expected if the resting potential were entirely attributable to a sodium diffusion potential. This and the saturation at high external sodium concentration suggests that the resting potential is largely the result of an inwardly directed sodium transport system. Externally applied 10^{-5} M and 5×10^{-4} M ouabain was without obvious effect on the resting potential or CP's. No other inhibitors have been tried.

(2.) *External cation substitution—calcium.* Calcium is the only cation besides sodium reported as being necessary for normal growth in the bathing solution of hydra (Ham *et al.*, 1956; Loomis and Lenhoff, 1956). To determine the effects of external calcium we replaced it in the bathing solution sequentially with magnesium, sucrose, and EDTA. The sucrose and EDTA solutions were obviously detrimental to the animal for the resting potential fell and failed to return to normal when solutions containing calcium were readmitted. It was therefore not appropriate to order randomly the test solutions used; rather they were presented in a sequence ordered according to increasing destructiveness to the animal. In no case did calcium-free medium have an obvious effect on CP amplitude or frequency. Resting potentials during calcium free periods and interspersed periods in normal solution are shown in Figure 7. There is no significant difference between the potential measured in normal solution and in the solution in which magesium replaced calcium. There is a significant decrease ($P < 0.01$) in the potential from the preceding control period when calcium is replaced by sucrose or by EDTA. EDTA, which will chelate any residual calcium or magnesium, is especially damaging and the transepithelial potential continues to decline even after EDTA solution has been replaced by normal solution. External calcium or magnesium seems necessary, at least in low concentration, to maintain the integrity of the animal.

There is a time delay between the beginning of external perfusion with calcium free solution and the onset of an effect on the resting potential. In the sodium substitution experiments there was a delay of only 1 to 3 seconds between the initiation of external perfusion with sodium free solution or readmission of normal solution and changes in the resting potential. At the onset of perfusion with calcium free sucrose solution there was a gradual decline in the resting potential rather than a rapid reduction. The resting potential decreases rapidly but after a delay of 15 to 20 seconds when calcium free EDTA was used. In both cases there are changes in the resting potential 1 to 3 seconds after readmission of calcium.

When normal solution is admitted following EDTA perfusion, the resting

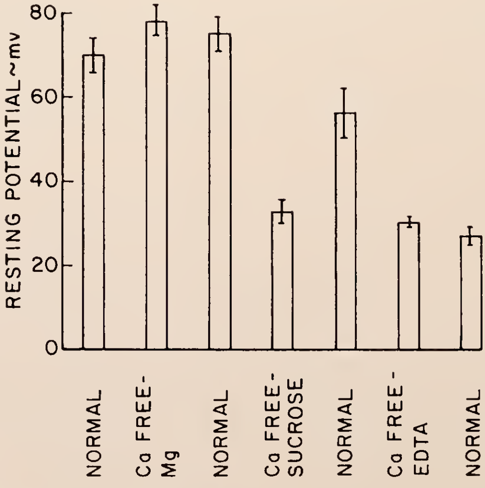

FIGURE 7. Dependence of transepithelial resting potential on presence of calcium and various calcium replacements. Data points were taken in the order plotted. Test periods were each 10 minutes long and only one test sequence was done with each animal. Each bar shows mean ± SE for 6 animals.

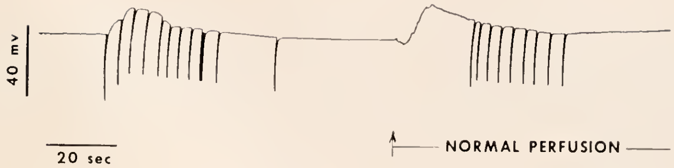

FIGURE 8. Transepithelial potential during CP bursts in Ca-free EDTA and immediately after admission of normal solution to the external perfusion chamber. Note the transient accompanying admission of normal solution. The internal perfusate was normal solution.

potential transiently increases and then drops back toward the level reached in the calcium free period (Fig. 8). The transient increase sometimes had oscillations on its leading edge reminiscent of those seen when sodium is readmitted following perfusion with sodium free solution. In most solutions the resting potential is stable or decreases during a burst of CP's; in EDTA solution the resting potential typically increased during CP bursts (Fig. 8). The significance of the increase in potential during CP firing in EDTA solution and following the readmission of normal solution after EDTA perfusion are yet obscure.

These results with calcium substitution indicate that external calcium is not directly involved in CP production but that calcium or magnesium is necessary in the bathing medium for the maintenance of the transepithelial resting potential. Judging by the time course of the response, the participation of calcium in the resting potential is less direct than that of sodium.

(3.) *External anion substitution.* The anions in the usual hydra culture solution are chloride and bicarbonate. The effect of these was first tested by replacing them both with sulfate which is generally assumed to be less permeant than chloride

TABLE II

Effect of external anion substitution on resting potential. For experiment 1, 8 animals were tested for 6 or 7 test sequences each, and for experiment 2, 6 animals were tested for 5 test sequences each. Each test solution was used for a 5 minute period. Normal solution was perfused interally during all tests. The difference between the measured values is not statistically significant in experiment 1 ($P > 0.1$)*. The values obtained in* $Cl^- + CH_3SO_3$ *and* $CH_3SO_3^-$ *are not significantly different* ($P > 0.1$)*, all of the other values are significantly different from one another* ($P < 0.01$*, except* $P < 0.05$ *for pair* $Cl^- + HCO_3^-/HCO_3^- + CH_3SO_3^-$)*. The difference in resting potential in normal solution* ($Cl^- + HCO_3^-$) *in the two experiments is indicative of the variations seen in experiments conducted with different groups of animals.*

Anions present	Resting potentials ± SE mv
Experiment 1	
$Cl^- + HCO_3^-$	71 ± 2
$SO_4^=$	67 ± 4
Experiement 2	
$Cl^- + HCO_3^-$	56 ± 3
$Cl^- + CH_3SO_3^-$	73 ± 3
$HCO_3^- + CH_3SO_3^-$	49 ± 2
$CH_3SO_3^-$	69 ± 2

(see *e.g.*, Stein, 1967). The replacement of both anions had no significant effect (Experiment 1 in Table II). We next replaced the two anions singly and jointly with methane sulfonate ($CH_3SO_3^-$), chosen because it is a moderately large, seemingly innocuous, monovalent anion (Milligan, 1965). In this series of experiments (Experiment 2, Table II) the resting potential was found to depend on the external anions, declining in the absence of chloride and increasing in the absence of bicarbonate.

(4.) *Change in impedance during ionic substitution.* In the first set of experiments the impedance and resting potentials were measured for 5 animals in normal solution immediately before perfusing with sodium or calcium free solution (tris or sucrose substitution). The impedance and transepithelial potential were then measured after 10 minutes in the ion substitution solution and then again 3 minutes after the beginning of external perfusion with the normal solution. In these experiments (A and B in Fig. 9) there was a significant change in potential but no statistically significant change in impedance due to the ion substitution. In a second set of experiments (C in Fig. 9) the column impedance and resting potential were compared for five animals when the animals were bathed with chloride-free methane sulfonate and with bicarbonate-free methane sulfonate, the solutions which gave the greatest difference in resting potential in Table II. In these experiments the test periods were five minutes long and each animal was alternately bathed in the two solution 3, 4 or 9 times; the series being ended when the animal pulled off the holder or pulled the current electrode through the body wall during column contraction. There was again no significant impedance change although

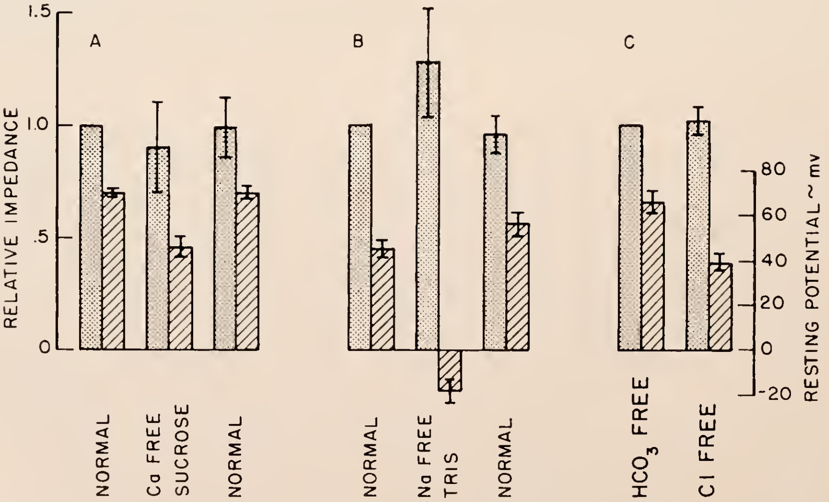

FIGURE 9. Effect of external ion substitution on body wall impedance. The left bar of each pair is the column impedance and the right bar is the mean resting potential. The impedance is shown relative to the first solution of each series. Means ± S.E. are plotted for five different animals in A, B and C. In all three cases the resting potential differs significantly in the two solutions ($P < 0.01$) but the impedances do not ($P > 0.1$). Test currents used were: A—0.2 μAmp at 1 Hertz, B—0.1 μAmp at 1 Hertz, C—0.2 μAmp at 5 Hertz. No internal perfusion was used in these tests.

TABLE III

Effect of anions on body wall impedance for one animal. This animal was tested for 9 test sequences; each test period lasted 5 minutes. The resting potential is significantly different ($P < 0.01$) in the two solutions whereas the impedance is not ($P > 0.1$).

Solution	HCO_3^- free, $CH_3SO_3^-$	Cl^- free, $CH_3SO_3^-$
Anions present	$Cl^- + CH_3SO_3^-$	$HCO_3^- + CH_3SO_3^-$
Potentials ± SE∼mv	76 ± 2	43 ± 1
Impedance ± SE∼Kohms	117 ± 7	124 ± 8
Current ± SE∼μAmp	0.67 ± 0.04	0.36 ± 0.03

the resting transepithelial potential differed markedly in the two test solutions. Table III gives the results from the single animal from which the most replicate measurements were obtained in the anion substitution series.

If a hydra has its mouth closed and the resting potential is constant there is no net current flow across the body wall. Active current due to ion transport mechanisms and gradients is balanced by passive current due to the voltage gradient. The active current component can be estimated by the ratio of transepithelial potential and transverse impedance. For hydra this ratio is a good estimate of short circuit current, the current measured when the transepithelial potential is held at zero (Table III of Josephson and Macklin, 1969; in this table the resting potential should be 22 ± 2 mv). The short circuit current calculated in this way for one experiment is shown in Table III. Chloride enhances and bicarbonate reduces transepithelial current.

(B.) *Internal ion substitution*

For the internal ion substitution experiments, two perfusion pipets were used which were driven by the same syringe pump. The animal was first perfused internally with normal solution and then the pipet used was replaced by one delivering the ion replacement solution. The animal was perfused internally with the test solution for 20 minutes and then again perfused internally with normal solution. Perfusion pipets were changed in 20 to 30 seconds.

Most internal ion substitutions were without obvious effect. There was no noticeable change in resting potential or CP's when the gut was perfused with sodium free solution (tris or potassium substitution), with solutions in which chloride and bicarbonate were replaced singly or together with sulfate or with solutions in which calcium had been replaced by sucrose. For periods of up to 2 hours the gut was perfused with normal solution which is potassium free without any effect on the resting potential or CP's, indicating that these potentials do not depend on potassium in the enteron fluid. When EDTA was used as a calcium substitute in the internal perfusate, the resting potential slowly decreased by 30 to 40 mV but recovered essentially to the pretest level within three minutes after normal solution was introduced into the enteron. In this case the effect of EDTA solution is not irreversibly destructive as it is on the outside of the animal.

Of greater interest was the effect of EDTA on the CP's. The CP is normally a monophasic, negative going spike superimposed on the positive resting potential. During internal perfusion with EDTA solution the CP magnitude gradually decreases and in many cases CP's became biphasic or of reversed polarity (Fig. 10).

CP recovery is rapid when normal solution is readmitted just as with the resting potential. The effects of EDTA perfusion were variable and with some animals the CP's became small but remained negative. One reasonable explanation for the variability in CP response to EDTA perfusion is that only a very small amount of divalent cation is required at the CP generating locus, an amount so small that it is but ineffectively removed by EDTA chelation in the face of diffusion from surrounding tissue.

(C.) *Osmotic pressure-effects*

Earlier it was hypothesized that the resting potential of hydra is the result of ion accumulating mechanisms used in osmoregulation (Macklin, 1967; Josephson and Macklin, 1969). One might therefore expect the size of the resting potential to reflect the osmotic stress faced by the animal. This was examined by determining the effects on transepithelial potentials of bathing media which were hyperosmotic to the normal medium. Increasing the osmotic concentration of the bathing medium somewhat above that of normal solution reduces the osmotic gradient between the tissues of the animal and its environment. In the first set of experiments (short term tests) the osmotic gradient was altered for 10 minute periods; in the second set of experiments (long term tests) the animals were kept in a solution with elevated osmotic concentration for two weeks before testing.

In the first short term tests the osmotic concentration of the bathing medium was varied by adding sucrose to normal solution (7.5 mosmol) to give calculated osmotic concentrations up to 67.5 mosmol. Animals did not fare well when exposed for 10 minute periods to these solutions presented in random order; the rest-

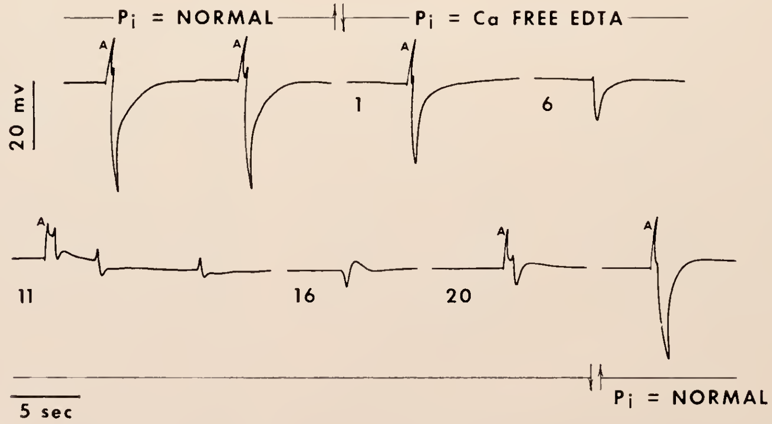

FIGURE 10. The effect on CP's of internal perfusion with Ca-free-EDTA. The animal was bathed in normal solution. Here the penwriter was capacitor-coupled so the records do not show the resting potential. The numbers below the record segments indicate the time in minutes since the onset of perfusion with Ca-free solution. The last CP was recorded 10 seconds after the perfusion pipet delivering Ca-free solution was replaced by one delivering normal solution. Some of the CP's shown were triggered by electrical stimuli, the rest were spontaneous. The stimulus artifacts preceding the triggered CP's are the upward deflections marked "A." Note that in the middle of the perfusion period the CP's were biphasic or principally positive. The CP shape seen at 11 minutes continued until 14 minutes.

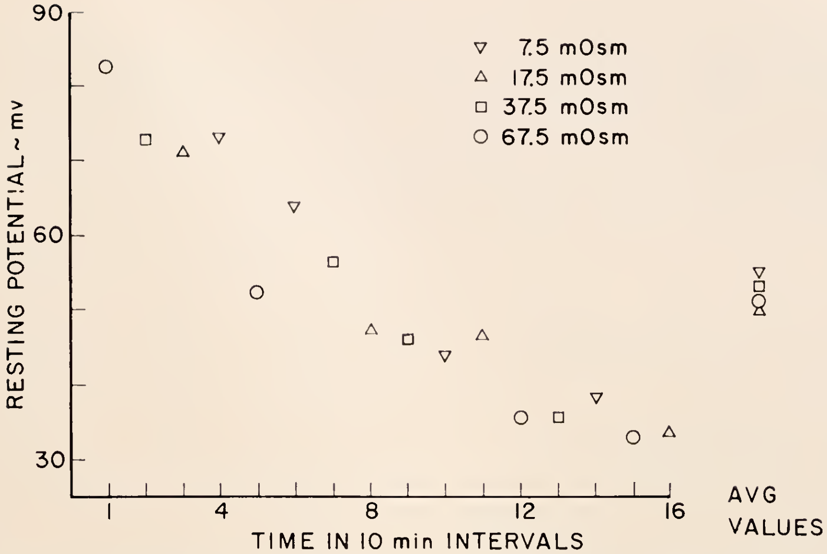

FIGURE 11. Destructive effect of high osmotic pressure on the resting potential of one animal. The resting potential continually fell when solutions of the indicated osmotic pressure were presented in random order for ten minute periods. Internal perfusate was normal solution which has a calculated osmotic pressure of 7.5 mosmole.

ing potential fell throughout the test series (Fig. 11). Toward the end of the series the animals were visibly moribund. Although not totally satisfactory because of the irreversible changes in the animals, these experiments did suggest that the resting potential was not greatly different during brief periods in solutions of differing osmotic concentration (see average values, Fig. 11). The test was then repeated omitting the 67.5 mosmol medium. There was no obvious degeneration when the 67.5 mosmol solution was omitted and reasonable resting potentials were maintained throughout the test series (Table IV). There was a small but not significant difference between the resting potentials in the different solutions. This result was not anticipated and it led to examination of the effects of long term exposure to a medium, with an elevated osmolarity.

For the long term test, twenty hydra were placed in each of two finger bowls, one bowl contained culture solution and the second bowl contained culture solution plus 10 mM/l NaCl. NaCl was used to increase the osmotic pressure rather than sucrose to avoid bacterial growth. The two cultures were fed normally and maintained in the two media for two weeks. During this time both cultures reproduced asexually at essentially the same rate—the population in culture solution increased from 20 to 28 animals and in culture solution plus 10 mM NaCl the population increased from 20 to 30. At the end of the two week period the animals were placed in randomly numbered vials. The vials were arranged in pairs, one containing an animal raised in normal solution and the other with an animal raised in the solution with an elevated osmotic strength. The sorting was done by an assistant who did not reveal the rearing solution of the animals to the authors until after they were tested.

TABLE IV

Effect of osmotic pressure on transepithelial resting potential. For the short term experiment, 6 animals were tested for 2 test sequences each, the solutions being presented in random order. For the two week experiment, animals were maintained in the rearing solution for two weeks prior to testing, then the resting potential for ten animals from each solution was measured. Test periods of 10 minutes were used for the short term experiment and 15 minutes for the 2 week experiment. The differences between the means in the short term experiment are not significant ($P > 0.1$) while the difference between means in the 2 week experiment is significant ($P < 0.01$).

	Solution osmotic pressure mosmol		Resting potential ± SE mv
	Rearing	Testing	
Short term experiment	7.5	7.5	72 ± 3
	7.5	17.5	69 ± 3
	7.5	37.5	67 ± 3
2 week experiment	7.5	7.5	65 ± 4
	27.5	7.5	49 ± 4

The animals were each placed on the test holder with normal solution on the outside and perfusing the gut. The recorded resting potential for each animal was determined as the average of readings taken every minute for the last 5 of the 15 minute test period. Measurements were made on ten animals from each culture, and the animals then identified. The animals grown for two weeks in the higher osmotic strength solution had a significantly lower resting potential than those grown in regular culture solution (Table IV). Thus animals accustomed to living in a higher osmotic concentration, and therefore a reduced osmotic gradient, have a lower resting potential. Hydra do not adapt to short term changes in osmotic gradient but there is long term adaptation.

DISCUSSION

Of the ions examined sodium is most directly involved in the transepithelial potential. The monotonic increase in the resting potential with increasing concentration of external sodium and the polarity of the potential suggest that at least part of the resting potential if not all of it results from inward movement of sodium—a transfer of sodium from the medium to the animal's tissue. The tissue sodium concentration in hydra is approximately 20 mM which is ten times the concentration in the usual bathing solution (Steinbach, 1963). A net inward movement of sodium must therefore involve an active transport mechanism. The slope of the potential versus external sodium concentration curve (Fig. 6) is less than the Nernst potential of 58 mv per decade and there is apparent saturation at high sodium concentration; both of these findings support the conclusion that sodium movement involves a transport system rather than solely passive diffusion. Although inwardly directed sodium transport appears to contribute to a potential across the epithelium and an inward current flow in animals whose transepithelial potential is clamped at zero (Josephson and Macklin, 1969), the active transport itself need not be electrogenic. For example, the transport mechanism might exchange sodium for another cation at one cell face and thus create a concentration gradient down which sodium passively diffuses at another cell face. This is the explanation originally offered for potentials across frog skin by Koefoed-Johnsen and Ussing (1958).

The rapidity of the potential change when the external sodium concentration is altered indicates that the ectodermal cell layer is the source of the potential change and indeed that it is the outer surface of the ectodermal cells at which the potential originates. It follows from this that sodium movement at the outer cell face involves charge separation, either by passive diffusion or the activity of an electrogenic pump. The sodium concentration in the ectodermal cells would have to be less than 1.5 mM for there to be a net movement of sodium into them by passive diffusion from the usual environment. If the transepithelial potential were due entirely to passive sodium movement across the outer cell face then the internal sodium concentration in the ectodermal cells would have to be about 0.015 mM judging by the external sodium concentration at which the potential changes sign in the tris substitution experiments (Fig. 6). Such a low sodium concentration is hard to reconcile with measured tissue concentrations. This line of argument supports the alternative possibility—*i.e.* an electrogenic pump at the ectodermal surface—but the question can not be settled until more is known about the intracellular sodium concentration and details of the potential profile across the body wall.

Although the resting potential declines when there is neither calcium nor magnesium in the bathing medium, the requirement for a divalent cation is less direct than for sodium as shown by the delay between the removal of calcium and the onset of the potential decline. Part of the decline in Ca^{++} free solutions may be due to cell damage. However there is little impedance change while the potential declines, indicating that the epithelia and the cells of which they are composed are remaining intact.

We think it particularly significant that the CP's become smaller and sometimes of reversed polarity when calcium is removed from the enteron by EDTA perfusion. Contraction pulses are probably generated by the basal surface of the ectodermal cells (Josephson and Macklin, 1969). The results of internal perfusion with EDTA suggest the following model for the genesis of CP's: They result from calcium moving inward across the basal membranes of the ectodermal cells, moving from the extracellular space on the mesogleal side into the ectodermal cells. The movement follows an increase in the calcium permeability of the basal membranes and the driving force is a gradient in calcium concentration from the extracellular space to the cell interior. When the enteron is perfused with EDTA the extracellular calcium concentration is lowered, reducing the gradient and the CP amplitude. During long perfusion periods the extracellular calcium concentration can be reduced sufficiently that the gradient is reversed; calcium now leaves the cells when the calcium permeability increases and the CP polarity is reversed. We are suggesting that CP's are calcium spikes, similar to those described for crayfish muscle fibers (Fatt and Ginsborg, 1959) and for barnacle muscle fibers (Hagiwara, Chichibu and Naka, 1964). Although CP's decline during initial test periods in Na free solution they do not do so in later periods (Fig. 5). Thus external sodium may influence CP amplitude, but its exact role is obscure.

Contraction pulses precede and are probably causally related to contraction of the ectodermal musculature. There is ample evidence in higher animals that the activity of muscle protein is controlled through the ambient calcium concentraction (Ebashi, Endo and Ohtsuki, 1969). It may be calcium influx during CP's which initiates contraction of hydra muscle fibers.

The transepithelial potential depends to some extent on the anionic composition of the bathing medium as is shown by the change in the potential when methane sulfonate replaces chloride or bicarbonate. The fact that both chloride and bicarbonate in the external medium can be replaced by sulfate with no significant change in the potential suggests that anion pumps, if they exist, are not major contributors to the transepithelial potential. It seems useful to see if the anion substitution results can be interpreted on the basis of passive anion movements acting as shunts for potentials developed by cation transport. The effectiveness of an anion species in shunting the transepithelial potential will vary with the conductance of the anion across the potential-generating structure. The potential changes in the methane sulfonate substitution experiments require the ionic conductances at the existing concentrations to decrease in the following order: $HCO_3^- > CH_3SO_3^- > Cl^-$. There is the difficulty that substitution among these ions was not reflected in a change in the column impedance. A possible explanation for this is that the barrier across which the potential is generated and at which ion shunting is effective makes up only a small part of the total transverse impedance, the remainder of the epithelia forming a large, passive impedance which is unaffected by short term changes of the external solution. This explanation is similar to the one which we proposed to account for the observation that there is little change in the transverse column impedance during CP's (Josephson and Macklin, 1969). Then we proposed that the CP generating membrane contributed only a small fraction of the total transverse impedance; now we are suggesting that the resting potential source also makes up but a small part of the transverse impedance.

In sum we have found that external sodium concentration is directly related to the transepithelial resting potential in hydra indicating a sodium transport mechanism. The effect of anions is as yet uncertain. Removing external divalent cations causes physiological deterioration, whereas removal of them from the gut with EDTA causes the CP's to change their shape. The latter result suggests that divalent cations are involved in CP production on the endoderm side of the CP generating membrane.

These experimental results support the following model for the maintenance of volume and osmotic equilibrium by hydra (see also Marshall, 1969). Sodium is transported from the outer medium to the gut, possibly in several steps. Anions passively follow the sodium. Because of the osmotic gradient, water enters cells of the epithelia from the outer bathing medium. In the animal, water movement is coupled to the movement of salt, either directly or through the creation of osmotic gradients, so that it too moves from the tissue to the gut. In this way water entering the animal is transported to the gut. The gut contents, which are hyperosmotic to the outer medium (R. Prusch and D. Benos, personal communication, Department of Biology, Case Western Reserve University; Marshall, 1969), are excreted by bulk flow to the environment, presumably through the mouth. This hypothesis is weakened by the observation that if an animal is transected, the cut surfaces heal over. A regenerate without either a mouth or a basal disc has a closed gut cavity but is still able to osmoregulate (Macklin, unpublished). However, the regenerate continues to contract spontaneously and may thus force fluid through adventitious openings in the body wall. The suggested mechanism by which hydra maintain both ionic and volume regulation is the same as the one recently described for another fresh water coelenterate, *Craspedacusta sowerbyi* medusae, by Hazelwood, Potts and Fleming (1970). Their conclusions derive from measurements of radioactive sodium and water transport rates and concentrations, whereas our conclusions are based primarily on electrophysiological measurements.

The ability of hydra to adapt to changes in osmotic pressure over a two week period but not within a ten minute period indicates that the effectiveness of the transport system can be slowly modified in response to environmental requirements, possibly by the synthesis of new carriers.

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SUMMARY

(1) The resting potential across the body wall of hydra varies monotonically with the external sodium concentration.

(2) Replacing bicarbonate and chloride, the normal external anions, with methane sulfonate changes the resting potential but not the transverse column impedance. If the anions are acting as passive shunts, the barrier across which the potential is developed must form but a small portion of the total transverse impedance.

(3) Changing the concentration of ions in the gut of hydra was generally without effect, but removal of calcium with EDTA caused the contraction pulses to become reduced or reversed in sign suggesting that these are calcium spikes.

(4) The resting potential changes in response to long term but not short term changes in the osmotic stress faced by the animal. It is proposed that the resting potential results from a sodium transport mechanism which is involved in osmotic regulation.

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AN AUTORADIOGRAPHIC ANALYSIS OF THE SPECIES SPECIFICITY DURING SPONGE CELL REAGGREGATION[1]

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The early studies of Wilson (1907) indicated that a mixture of sponge cells from two species would separate to form aggregates which were species-specific. Mixed suspensions of cells were seen to form small spherical aggregates which then coalesced with other aggregates of the same species. More recently, the specificity of aggregation has been brought into question by a number of investigators (Curtis, 1962, 1970; Sara, Liaci, and Melone, 1966a, 1966b; Sara, 1968; MacLennan, 1970; Humphreys, 1970a). Sponges have been observed to form bispecific mixtures upon reaggregation after a variety of treatments. This random association of cells may result from a number of causes. For example, dissociation procedures might remove molecules from the surface of the cell which would otherwise confer specificity to it (Humphreys, 1963; Moscona, 1963). Cellular injury might occur due to dissociation or to culturing procedures which could affect aggregation (Curtis, 1962). There is a good possibility that heterospecific cells could be trapped passively in aggregates during the early phases of aggregation. Finally, a cell specific mechanism for aggregation might not exist for many species.

Recently, a method has been developed which shows the species- and tissue-specific nature of freshly dissociated embryonic vertebrate cells. Roth and Weston (1967) developed an "aggregate collection" procedure which has been shown to be a useful tool for analyzing cell specificities in aggregation. The method utilizes monospecific aggregates which have recovered from the stress of dissociation. These aggregates are secondarily confronted with freshly dissociated cells. The results of Roth (1968) have shown that embryonic chick cell aggregates will selectively collect cells of their own genetic or histological type. Because of these results, it is of interest to ascertain whether this same phenomenon can be demonstrated with sponges. The aggregate collection method in this study has been used as a device to show the presence of a specificity which has not been otherwise demonstrated in sponges.

Curtis (1962) pointed out that most studies on specificity had utilized the color of the sponge as the only criterion for species identification in cell aggregates. In many sponges only a small percentage of cells contain pigment; therefore, these studies afforded no means of monitoring behavior in a majority of the cells. The use of radioactive labels is a useful way to overcome this identification problem because the label, when used in small quantities, can allow for a precise identification of all cells without impairing the normal behavior of the cells.

The present study utilizes the aggregate collection method for a series of experiments in which two unlabeled aggregates are placed into the same suspension of labeled cells. The aggregates compete for the same cells for the same period of time. One aggregate is of the same species as the cells in suspension and the other aggregate is of a different species.

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MATERIALS AND METHODS

Five species of sponge were used for this study: *Haliclona variabilis, Haliclona viridis, Tedania ignis, Homaxinella rudis,* and *Dysidea crawshayi* (DeLaubenfels, 1950). These species were chosen from more than 60 species in Bermuda because they were easily obtained, aggregated well, and were able to survive well under laboratory conditions. They also represent a diversity in taxonomic relationship. Although they are all members of the Class Demospongiae, two are within the same genus; *Haliclona, Tedania,* and *Homaxinella* are in separate Orders of the Subclass Monaxonida; and the Genus *Dysidea* is most distantly related to the others since it is in the Subclass Keratosa.

The sponges were collected by hand from Harrington Sound, Bermuda. They were transferred individually to glass jars under water. The jars were sealed and immediately returned to the laboratory. All experiments utilized the fresh material within two hours following collection.

Preparation of the aggregates

Humphreys' procedures (1963) for the dissociation and aggregation processes were used with the following minor modifications. Small pieces (1 cm^3) were cut from a sponge and washed in Millipore filtered seawater. The tissue was pressed through #24 mesh bolting cloth and the dissociated cells were collected in calcium-magnesium–free seawater (CMF-SW). The cells were washed twice in CMF-SW with slow speed centrifugation. They were then resuspended in Millipore filtered seawater containing streptomycin sulfate and sulfadiazine (0.1 mg/ml of each) (MSS-SW). The suspension was diluted to a cell concentration of 5×10^6 cells/ml and 3 ml aliquots of cell suspension were placed into 10-ml culture dishes. These were placed into moisture chambers and the suspensions were rotated at 80 rpm on a shaker at 24° C. Aggregates were harvested after six hours and were selected so that the size of aggregates used in each experiment was initially equal.

Preparation of labeled cells and collection procedures

Cells were disaggregated and washed as described before. They were then resuspended to a final concentration of 20×10^6 cells/ml. in CMF-SW plus ^{3}H-leucine (Schwartz) at a final dilution of 1 μC/ml. ^{3}H-leucine was used instead of other radioactive labels because the cells incorporated it rapidly as opposed to ^{3}H-thymidine which was taken up only slowly by these adult cells. The cells were rotated in plus label for four hours. During this time aggregation was inhibited by the CMF-SW in which the cells and label were suspended. Cell counts after this time indicated that most cells had remained intact and autoradiographs of these cells showed that virtually every cell had incorporated label. After this four hour period, the cells were washed three times in MSS-SW, and then resuspended in MSS-SW to a concentration of 5×10^6 cells/ml. Two aggregates selected for uniform size were placed into each suspension of labeled cells. One aggregate was of the same species as the cell suspension and one was of a different species. These suspensions were rotated at 80 rpm at 24° C in moisture chambers. Replicates were fixed at 6 and 18 hours in Bouin's fixative where they were stored until processing. All combinations of the five species were tested and the results were analyzed by autoradiography.

Histological processing

The aggregates were dehydrated, embedded in paraffin and sectioned at 4 μ. Sections were mounted serially on albumin-coated slides, dried, cleared, and rehydrated to distilled water. They were then soaked for five minutes in cold (0° C) trichloroacetic acid to remove any remaining unbound label. The slides were then rinsed twice in distilled water and processed for autoradiography. The procedures of Kopriwa and Leblond (1962) were followed and were carried out in total darkness. The slides were dipped into Kodak NTB-2 photographic emulsion at 40° C for 3 seconds, drained and dried for 2 hours at 28° C. Coated sildes were stored in dry, light-tight containers at 4° C and were exposed for six weeks. The emulsion was developed with Dektol, stained in 0.1% nuclear fast red and counterstained briefly in 0.2% indigo carmine in saturated aqueous picric acid. Grains were counted under oil immersion using an ocular grid to delineate an overall area of 40 μ^2 at this magnification. Photographs were taken with a Leitz Ortholux camera.

RESULTS

Incorporation of label

The primary objective in labeling the cells was to place a radioactive tag on proteins of each cell. For all five species it was determined that better than 95% of the dissociated cells picked up ^{3}H-leucine under the conditions used. The amount of label picked up by single cells was, however, heterogeneous. In autoradiographs, most cells within a species had from four to fifteen grains over them, but for each species there was a small population of cells which incorporated such a large amount of label that the number of grains was too large to count. The distribution of these cells after collection onto unlabeled aggregates was random although the heavily labeled cells were a constant percentage of the total number of collected cells for a species. No effort was made to determine whether these heavily labeled cells were all of a single histological cell type.

The unlabeled collecting aggregates were closely examined to determine the "background" level in the autoradiographs in areas devoid of labeled cells. Large section areas such as those marked "UA" on Figure 1 were examined for grain distribution. When the number of grains was determined on a per-cell basis, the average for each species was 1.6 grains/cell or less. Table I shows the average number of grains appearing over an entire 40 μ^2 area in the unlabeled aggregates. As can be seen, the number of grains appearing over aggregate sections is related to the kind of cell suspension to which the aggregate was exposed. Thus, more label was introduced into unlabeled aggregates by exposure to *Haliclona variabilis, Tedania ignis*, or *Dysidea crawshayi* cell suspensions than by exposure to *Haliclona viridis* or *Homaxinella rudis* suspensions. This introduction of label was essen-

TABLE I

Background grain counts in unlabeled aggregates derived by exposure to ^{3}H-leucine labeled cell suspensions. Each number represents at least eight counts of 40 μ^2 areas from 6- and 18-hour aggregates

Unlabeled collecting aggregate	Labeled cell suspension				
	Haliclona variabilis	*Haliclona viridis*	*Tedania ignis*	*Homaxinella rudis*	*Dysidea crawshayi*
Haliclona variabilis	53	5	40	13	52
Haliclona viridis	47	17	27	10	49
Tedania ignis	59	15	63	17	75
Homaxinella rudis	63	11	60	18	68
Dysidea crawshayi	50	12	46	16	66
Average	54	12	47	15	62

tially uniform for each kind of cell suspension; that is, *Haliclona variabilis* collecting aggregates did not pick up any more background label from a *Haliclona variabilis* cell suspension than did any of the other four collecting aggregates. Because this incorporation from the cell suspensions was low, at random and essentially uniform throughout, the grains over collection aggregate cells are considered to be "background" for purposes of this study.

It was important to determine whether cells specifically labeled with ^{3}H-leucine would continue to be identifiable for the duration of the experiment. Collecting aggregates from each of the five species were fixed after 6- and 18-hour exposures in each of the five labeled cell suspensions. The autoradiographs of sections from these aggregates were compared by counting the number of grains over cells in labeled and unlabeled areas. In all cases, the small population of cells with exceptionally heavy accumulation of label were excluded from the counts. Table II summarizes the data from this study. First, it can be seen that the number of grains over labeled cells is always much greater than over background cells. By inspection of corrected grain counts in Table II, one can see that there is no significant difference in the number of grains appearing over the comparable cells after 6 and 18 hours. If loss of label due to metabolic turnover were significant, it would be expected that the number of grains over labeled cells would decrease with time, while the background would increase. This was not the case, as is shown in Table II. The difference between background and labeled cells is at least five fold in all cases. This difference is just as apparent after eighteen as after six hours. Because of this large and substantial difference it was possible to identify with great confidence, on a cell to cell basis, the cells in aggregates that were secondarily incorporated from labeled cell suspensions.

Observations on mixtures of cells in suspension

A preliminary study was made in order to observe the aggregation behavior of mixed cell suspensions. On the assumption that mechanisms for species specificity during cell reaggregation might be most pronounced between species with distant

TABLE II

Average grains/cell in labeled and unlabeled areas. Each figure is the average grain number per average cell number per 40 μ^2 area. Heavily labeled cells are excluded

Unlabeled collecting aggregate	Grains/cell in labeled areas		Background/cell in background areas		Corrected grains/cell in labeled areas	
	6 hr.	18 hr.	6 hr.	18 hr.	6 hr.	18 hr.
Haliclona variabilis	10.7	9.9	1.6	1.3	9.1	8.5
Haliclona viridis	5.8	6.2	0.8	0.9	5.0	5.3
Tedania ignis	14.9	15.0	1.3	1.3	13.6	13.7
Homaxinella rudis	10.7	11.2	0.6	0.7	10.1	10.5
Dysidea crawshayi	15.0	16.0	1.3	1.4	13.7	14.6

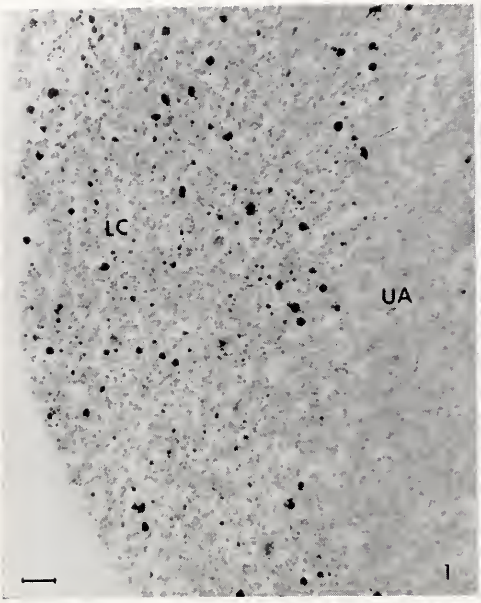
taxonomic relationships and less effective between closely related species, these cell suspensions were given special attention. However, no such correlations were observed in the heterospecific combinations used in this study. The same level of species selectivity was observed between closely related species (*Haliclona variabilis—Haliclona viridis*) as was observed between more remotely related species.

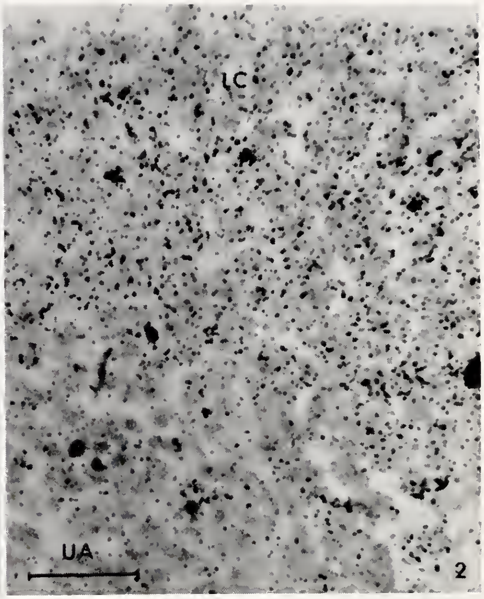
When unlabeled cells from any two of the species used were washed with CMF-SW and then mixed in suspension, they were often observed to clump together heterospecifically. After one to two hours, it was no longer possible to determine whether the aggregates were being formed heterospecifically. By this time, each aggregate had assumed a macroscopically recognizable color of one of the two species.

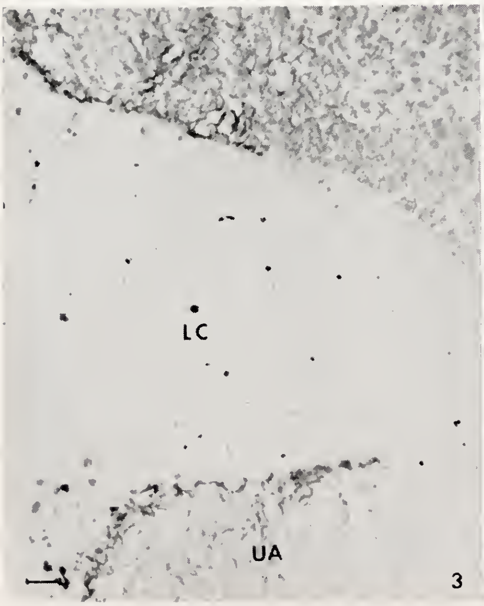
Aggregates formed from mixtures of dissociated cells from two species in which one cell type was radioactively labeled often contained mixtures of labeled and unlabeled cells. For example, when labeled *Haliclona viridis* cells were mixed with unlabeled *Homaxinella rudis* cells and permitted to aggregate for six hours, the green (*Haliclona viridis*) aggregates contained mostly labeled cells with a few unlabeled cells, whereas the red (*Homaxinella rudis*) aggregates were primarily unlabeled, but contained a scattered proportion of labeled *Haliclona* cells. From these preliminary results, it is evident that there is some heterospecific mixing and incorporation of cells even though the aggregates which result are predominately of one species. These results, however, do not show whether this mixing is due to an absence of any species specificity or to a loss of specificity. Also, they do not show whether the clumping which occurs is true aggregation or whether it is a nonspecific response to cellular injury.

Collection of labeled cells by unlabeled aggregates

The pattern of collection of labeled cells by heterotypic aggregates was quite different than that of the homologous collection of cells. Figure 1 is an autoradiograph that shows darkly labeled cells of *Dysidea crawshayi* after collection by a *Dysidea crawshayi* aggregate. As can be seen, the labeled area is integrated within the unlabeled cells of the aggregate. This appearance is characteristic of all five homotypic combinations. At first, the aggregates became almost completely surrounded by labeled cells. Between six and eighteen hours, there is a progressive







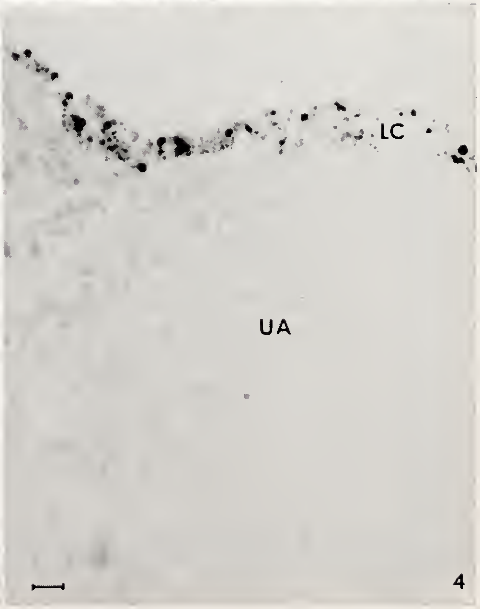


FIGURE 1. Eighteen hour homotypic collection of labeled *Dysidea crawshayi* cells (LC) to an unlabeled *Dysidea crawshayi* collecting aggregate (UA); scale = 10 microns.

FIGURE 2. Eighteen hour homotypic collection of labeled *Homaxinella rudis* cells (LC) to an unlabeled *Homaxinella rudis* aggregate showing the mixing of labeled and unlabeled cells; scale = 10 microns.

FIGURE 3. Eighteen hour heterotypic collection of labeled *Haliclona variabilis* cells (LC) into a folded area of a *Haliclona viridis* collecting aggregate (UA); scale = 10 microns.

FIGURE 4. Eighteen hour heterotypic collection of labeled *Haliclona variabilis* cells (LC) to the surface of an unlabeled *Homaxinella rudis* aggregate (UA); scale = 10 microns.

TABLE III

The distribution of labeled cells on collecting aggregates. (M) mixing of labeled and unlabeled cells. (S) adhesion to the surface of collecting aggregates but no mixing. (O) no labeled cells on aggregates surface. This table summarizes three replicates of aggregate collection experiments

Unlabeled collecting aggregate	Labeled cell suspension				
	Haliclona variabilis	*Haliclona viridis*	*Tedania ignis*	*Homaxinella rudis*	*Dysidea crawshayi*
Haliclona variabilis	MMM	OOS	OOS	SSM	SSS
Haliclona viridis	OOS	MMM	OOO	SSS	OOS
Tedania ignis	SSS	OOS	MMM	SSS	OOS
Homaxinella rudis	OSS	OOS	OOS	MMM	OOS
Dysidea crawshayi	OOS	OSS	OOS	SSS	MMM

mixing of labeled cells with the unlabeled cells of the collecting aggregate. Table III shows the overall results of this autoradiographic examination. The sections were examined for the presence of label and were scored according to three categories: "M," mixing (meaning that the labeled cells were mixing into the unlabeled aggregate), "S," surface adhesion but no mixing, and "O," no collection of labeled cells by aggregates.

Each of the 25 permutations of combinations among aggregates and dissociated cells was repeated three times. In all three replicates, homotypic collections showed labeled cells mixed in among unlabeled cells of the aggregate as is shown by the distribution of autoradiographic label in Figure 2 and in Table III. In only one heterotypic combination was any mixing observed. The exception was found in one of three replicates in which a *Haliclona variabilis* aggregate collected cells of *Homaxinella rudis.* In this case, labeled cells were found in the core of the aggregate. The pattern of aggregate formation for *Haliclona variabilis* and *Haliclona viridis* is such that foreign cells can occasionally be trapped passively in the interior of the aggregate. During the first few hours of aggregation, these species first form small spherical aggregates which fuse to form a flat sheet. This sheet then folds up to form a large sphere. Any cells resting on the surface of such an aggregate can be passively trapped to the inside during the formation of a sphere. Figure 3 shows an example where this has occurred. Cells of *Haliclona variabilis* are partially trapped in the folds of a *Haliclona viridis* aggregate and still there is no mixing. The exceptional case in Table III may have been the result of this kind of entrapment.

Figure 4 shows a typical example of a combination in which heterotypic cells have been collected on the surface of an aggregate. In Figure 4, labeled cells of *Haliclona variabilis* have adhered to the surface of an aggregate of *Homaxinella rudis.* In this, and the other cases such as this, a few labeled heterotypic cells were observed at the periphery of unlabeled aggregates, but the labeled cells were not tightly bound to the unlabeled aggregate. In fact, in many cases where labeled heterotypic cells were present, there was the appearance of a rejection or a separation of the labeled mass from the surface of the unlabeled aggregate as can be seen in Figure 4. With the possible exception of the one case already mentioned, mixing of labeled heterotypic cells with unlabeled collecting aggregates was not observed.

TABLE IV

Collection of heavily labeled cells per 0.2 mm² area on collecting aggregates at 6 and 18 hours of aggregation. Each number represents the average of nine counts

Unlabeled collecting aggregate	Homotypic collection		Heterotypic collection	
	6 hr	18 hr	6 hr	18 hr
Haliclona variabilis	48	53	15	5
Haliclona viridis	—	—	8	1
Tedania ignis	90	97	31	27
Homaxinella rudis	32	24	2	4
Dysidea crawshayi	40	49	13	9

Most of the cells were collected by the aggregates during the first few hours of aggregation. A study of the sections was carried out to determine whether the number of cells collected increased between six and eighteen hours, or whether collected cells might be lost from the collections. Table IV summarizes the results from counting the number of heavily labeled cells appearing per aggregate section. The heavily labeled cells were easy to recognize and although they constituted only a small proportion of the total number of cells for a species, this proportion was constant for a species. Each number represents the average count for at least nine sections. The sections to be counted were chosen from the largest cross sections of an aggregate and the average count of three adjacent sections was used for each of three replicates. The size of the collecting aggregate varied for each experiment; thus, in order to standardize the counts, the area of the central section of an aggregate was determined. Each figure in Table IV represents the number of heavily labeled cells per 0.2 mm² area in an aggregate section. This number was more difficult to ascertain in heterotypic combinations since the total number of cells collected was small and often these cells were found in surface patches on the collecting aggregates. For the latter cases, counts were made on the three adjacent sections which contained the greatest number of heterotypic cells. *Haliclona viridis* was not included in this study because its percentage of heavily labeled cells was less than 5% of the total number of cells labeled and the values obtained were to low to provide any meaningful data on cell loss. A comparison of 6- and 18-hour radioactive cells in Table IV indicates a trend for cells to be lost from the heterotypic collections and a trend for cells to be added to homotypic collections.

DISCUSSION

This study demonstrates the presence of a species recognition mechanism for dissociated cells of five species of sponge. The data presented here strongly indicate that the specificity of cell recognition, as measured by selective adhesion, may be temporarily weakened by cell dissociation, but within a few hours, species specficity is reestablished and provides an effective isolating mechanism at the level of cell to cell interactions.

Cells were observed to mix nonspecifically during the early stages of aggregation. It would appear that the early stages of aggregation are somehow different from the processes taking place later in aggregation. Moscona (1965) has described this as the "primary stage" during which random cell associations take place. Sheffield and Moscona (1969) and Sheffield (1970) have studied the primary phase of embryonic chick retina aggregation and have found a random association of histotypic cells during the first one to two hours of aggregation formation. Roth (1968) demonstrated this primary phase indirectly. Using embryonic chick and mouse cells and the aggregate collection system, he found that many more labeled heterotypic cells were picked up by aggregates when unlabeled freshly suspended homotypic cells were included in the suspension, than when heterotypic cells alone were present. There was a random association between the homotypic and heterotypic cells and a specific association between the homotypic cells and the collecting aggregate. This primary phase has been shown in a number of well known studies (Townes and Holtfreter, 1955; Moscona, 1957; Steinberg, 1962), but in these papers, stress was placed on the process of sorting out which demonstrated the return of specificity that was lost or latent during the dissociation procedures.

Nonspecific associations could occur for several possible reasons. Cells may lose specific combining or reactive groups on the cell surface as a result of dissociation; the cells may stick together in response to injury incurred during dissociation; or there might not be a mechanism for specificity during aggregation. Unless cells subsequently sort out, it is difficult to ascertain whether a specific mechanism of recognition exists. Aggregates which do not sort out may normally have a specific recognition system, but experimental conditions might be such that this recognition can not be expressed.

Most reports on the nonspecific aggregation by sponge cells (Curtis, 1962, 1970; Sara *et al.*, 1966a, 1966b; MacLennan, 1970; Humphreys, 1970a) have been based on observations of cells during the early phases of aggregation when an inability to recognize homotypes occurred. This has been observed in the present study. It is possible that these examples represent cases where there is no mechanism for cell recognition. However, one of these reports (Sara *et al.*, 1966b) describes the formation of "mosaics" following bispecific aggregation. It is likely, as shown in the present study, that the mixed aggregates are the result of the primary stage in which no recognition mechanism is present. The cells might re-acquire and demonstrate specificity as in the present results. This acquisition of specificity might be analogous to the sorting out phase which has been observed in vertebrate tissues.

The cases where nonspecificity has been observed in sponges may well be due to the loss of specific surface recognition groups. Studies on enhancement of aggregation (Humphreys, 1963, 1970a; and Moscona, 1963, 1968) have shown that for several species of sponge, a factor can be isolated which enhances aggregation species specifically. These studies indicate that a glycoprotein, lost during dissociation in CMF-SW or Pronase, must be replaced or resynthesized before the cells are able to reaggregate.

Cell injury may play an important part in nonspecific aggregate formation. The cells of *Homaxinella rudis* perhaps best demonstrated this possibility. These cells loosely collected onto heterotypic aggregates in greater proportion than cells of any other species tested. Dissociation of cells with CMF-SW permitted subsequently a greater incidence of mixed agglutinations than did more gentle washing with MSS-SW (the term "agglutination" is used here to distinguish loose cell masses which are easily broken up by pipeting, as opposed to "aggregates" in which adhesions are tighter and tend to resist breakdown by pipeting). As was the case in the early studies of Galtsoff (1929), agglutination often resulted in cytolysis of cells, indicating that cellular injury might have been the factor which caused this agglutination. In their studies on mixed aggregates, both Sara *et al.* (1966b) and Curtis (1962, 1970) used EDTA to dissociate cells. Ball (1966) and Moscona and Moscona (1967) have shown that EDTA has a toxic effect on vertebrate cells. If EDTA were injurious to the sponge cells as Humphreys (1970b) has observed, then the response of the cells might have been a nonspecific "injury" agglutination. If the cells were unable to recover from the treatment, then the nonspecific masses would have remained mixed. Likewise, CMF-SW, trypsin, and other treatments might cause some injury to the cells which could lead to nonspecific agglutinations or aggregations.

The present results show that cells are collected by homotypic aggregates. By six hours, much of this collection is complete. Humphreys (1970b) has pointed out that it might be necessary for only one of two entities (in this case the aggregate) to have a specificity in an aggregate system. The freshly dissociated cell does not have the ability to form a specific association with other cells, but it might respond to an aggregate which has regained specificity. During this time, however, heterotypic aggregation is also taking place. The present results show that the number of cells picked up heterotypically is far less than homotypic collections. This indicates that even if injury were a factor, the adhesions formed by homotypic cells are stronger or more permanent.

The most important adhesions in the present results are those which form first. The vast majority of cells collected to an unlabeled aggregate actually adhere to the labeled cells that were first collected for the simple reason that only one layer can be formed between the aggregate and the collected cells. Subsequent adhesions to collected cells are independent of aggregate influence. Therefore, the specificity demonstrated involves the initial adhesion of cells only. For this reason, the absolute number of cells collected by an aggregate does not reflect the specificity of the cells for the aggregate, but does reflect the stability of the initial adhesions. Adhesive stability may be important in the process which was demonstrated by these experiments. If it can be assumed that layers of cells will continue to add to the collected cells, and given that the shearing force of rotation is present, then the thickness of the collected cell layer reflects the stability of the adhesion between the collecting aggregate and the first layer of cells. If the cell-aggregate adhesion is not as strong as the cell-cell adhesions, or if that former adhesion is gradually lost, then the entire layer of collected cells would tend to peel away from the collecting aggregate as a result of shear forces. The loose patches of heterotypic cells which were observed in these experiments may be indicative of this process. On the other hand, if the cell-aggregate adhesion becomes just as strong as the intra-aggregate adhesions, then it would be expected that cells could be added to the surface continually until shear forces would prevent further addition.

The present results show that the most stable configurations are between homotypes and that the original collecting aggregate surface becomes indistinguishable as additional homotypic cells are added and move into the aggregate. Therefore, even though nonspecific adhesions may occur early in the aggregate collection process, these adhesions are not as strong nor as stable as those between a cell and an aggregate of the same species. With time and recovery from dissociation, heterospecific cells, if collected, will progressively be lost from a collecting aggregate of a different species.

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SUMMARY

Unlabeled sponge aggregates were placed into suspensions of radioactively labeled sponge cells. All combinations of five species (*Haliclona variabilis, Haliclona viridis, Tedania ignis, Homaxinella rudis,* and *Dysidea crawshayi*) were used for aggregate collection experiments designed to test for species specificity of adhesion. Preliminary experiments had shown that freshly disaggregated cells from any two of the species would co-mingle during early aggregation. The aggregate collection system, however, showed the presence of adhesive specificity for all five species. Labeled and unlabeled cells became mixed when an unlabeled aggregate collected radioactive homotypic cells. Very few labeled cells were collected and mixing was not observed in heterotypic combinations.

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CORTICOSTERONE PHASES A CIRCADIAN WATER-DRIVE RESPONSE TO PROLACTIN IN THE SPOTTED NEWT, *NOTOPTHALMUS VIRIDESCENS*[1]

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Several responses to prolactin have recently been found to vary in a circadian manner. Injections of prolactin may be considerably more effective, or produce entirely different results, at one time of day compared to another. A daily variation in responsiveness to prolactin was first observed in a migratory sparrow wherein prolactin injections given daily at midday of a 16-hour photoperiod induced large increases in body fat, whereas injections given early in the day caused losses in fat stores (Meier and Davis, 1967). Subsequently, daily variations in fattening responses to prolactin have been reported in fish (Lee and Meier, 1967; Meier, 1969; Mehrle and Fleming, 1970), frogs, and lizards (Meier, 1969). Other daily variations in responses to prolactin include locomotor activity in a migratory sparrow (Meier, 1969), growth in fish (Lee and Meier, 1967), pigeon cropsac stimulation (Burns and Meier, 1971; Meier, Trobec, Joseph and John, 1971), and inhibition of frog tadpole metamorphosis (Breaux and Meier, 1971).

In all these studies it is clear that the daily variations in responses to prolactin are synchronized by the photoperiod. In addition, the photoperiodic effect on the rhythm of tissue sensitivity seems to be mediated by the interrenal system. In animals on continuous light, injections of adrenal steroids phase circadian rhythms of fattening responses to prolactin in fish, lizards and birds that simulate the phasing effects of the photoperiod (Meier, Trobec, Joseph and John, 1971; Meier and Martin, 1971). Circadian variations in the pigeon cropsac response to prolactin are also phased in pigeons on continuous light by injections of an adrenal steroid (Meier, Trobec, Joseph and John, 1971).

Another of the many interesting effects of prolactin is an ability to promote migration to water in the immature, terrestrial stage (red eft) of the spotted newt, *Notopthalmus viridescens* (Chadwick, 1940; Grant and Grant, 1958). The red eft water drive has served as the basis for prolactin assays (Grant, 1959), particularly with respect to the lower vertebrates (Grant and Pickford, 1959). The purpose of the studies reported herein was to test whether there are daily variations in the effectiveness of prolactin to induce the red eft water drive.

MATERIALS AND METHODS

Efts of the spotted newt, *Notopthalmus viridescens*, were obtained from Mr. Lewis Babbitt, Petersham, Massachusetts. The efts ranged in weight from 0.5 to 1.5 gm. Following their arrival in June, 1970, they were maintained in aquaria which contained an area of open water and an area of fine moist gravel and moss. An abun-

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TABLE I

*Daily variations in the Water drive response to prolactin**

Time of injection (Hours after onset of light)	No. of responses	Time to water (Mean no. of hours)	Total dose (Mean in μg)
0	4 of 7	389	11.0
8	6 of 8	341	8.6
16	5 of 6	155	4.6

* The photoperiod was 16 hours (0800–2400).

dance of miniature wing fruit flies (*Drosophila*) were supplied for food. The temperature ranged from 25°–28° C. Incandescent light (50 lux at the surface of the gravel) was used to produce a 16-hour photoperiod during the holding period and during the first experiment. Continuous light was given for the second experiment.

In the first experiment, intraperitoneal injections of ovine prolactin (1 μg/eft in 0.01 ml 0.65% saline) were initiated on 8 July and continued on alternate days until 20 July when the dose level was increased to 2 μg/eft/day. The injections were continued until the efts migrated to water or until 24 July when the experiment was terminated. The injections were made at 0, 8 or 16 hours after the onset of a 16-hour photoperiod (0700–2300). Twenty-one efts were divided among the three treatment groups.

In the second experiment, the efts were acclimated to continuous light for one month before the injections were begun. Corticosterone injections (1 μg/eft in 0.01 ml 0.65% saline) were initiated on 6 October and continued on alternate days. Ovine prolactin injections (2 μg/eft in 0.01 ml 0.65% saline(were initiated on 8 October and continued daily until the efts migrated to water. Corticosterone injections were made either at 0800 or at 2400. Each corticosterone group received prolactin injections at 0800, 1600, or 2400. Another group received prolactin, alone, at 2400. All groups were composed initially of 5 efts. Two efts died during the experimental treatment.

The criteria used for judging the water-drive response were similar to those described by Grant and Picksford (1959). Efts in the terrestrial stage stay in moist areas, but they are found in water only for short periods. Efts that move permanently (for at least 5 days) to water were judged to have migrated. The aquatic adult was further distinguished by a strongly keeled tail and by the development of an olive pigmentation.

RESULTS

There is a considerable difference in the effectiveness of prolactin to cause migration to water depending on the time of injection during a 16-hour photoperiod (Table I). Injections at the end of the photoperiod are most effective. Not only are there more positive responses in the late group (5 of 6) than in the midday (6 of 8) and early (4 of 7) groups, but the time it took for the responding efts to move to water was shorter. The late group averaged 155 hours to water, whereas the midday and early groups averaged 341 and 389 hours, respectively.

TABLE II

*Corticosterone phases the circadian variations in water drive response to prolactin**

Time of corticosterone injection	Time of prolactin injection (Hours after corticosterone)	Time to water (Mean no. of hours)	Total dose of prolactin (Mean in μg)
0800	0	143	12.8
	8	106	10.0
	16	260**	21.5
2400	0	148	13.0
	8	93	8.4
	16	230	19.0
None (saline at 2400)	(2400)	171	14.8

* Animals maintained in constant light.

** One eft failed to respond.

According to an analysis of variance for variable hours, the time to water of the late group was less ($P < 0.05$) than those of the early and midday groups. The early and midday groups did not differ significantly from one another. For statistical purposes, only those efts that went to water were considered.

The second experiment was designed to test whether the injections of corticosterone could phase a circadian rhythm of sensitivity of the water drive response to prolactin (Table II). Examination of the results of both groups receiving corticosterone reveals that injections of prolactin given 8 hours after the time of corticosterone injections are most effective in promoting migration to water (106 and 93 hours), and injections 16 hours after corticosterone are least effective (260 and 230 hours). With only one exception, all the times to water in the 2 groups of efts receiving prolactin 8 hours after corticosterone were shorter than the times to water of the efts receiving prolactin 16 hours after corticosterone. Intermediate values were obtained in the two groups of efts receiving prolactin at the same time as corticosterone (148 and 146 hours) and in the group receiving prolactin, alone (171 hours).

According to a least-squares analysis of variance, the responses varied according to the time of prolactin injection relative to corticosterone ($P < 0.01$), whether corticosterone injections were given at 0800 or 2400. There were no differences between the 2 corticosterone-treated groups. The time responses to prolactin fit quadratic curves ($P < 0.05$).

Using the second experiment as a guide, we tested whether handling alone might simulate the effects of corticosterone injections and account for the temporal variations in responses to prolactin. Because of a limited number of animals, the test was restricted to the 16-hour interval in efts maintained on continuous light in May. Corticosterone (1 μg) or saline injections were made on alternate days at 0800 and prolactin (1 μg) was given daily at 2400. The 3 efts receiving saline injections migrated to water in 120, 144, and 168 hours. Two of the 3 efts receiving corticosterone migrated after 240 and 264 hours, and the remaining eft had not migrated after 312 hours when the test was terminated.

Although saline-injected controls were not available for every temporal pattern of hormones tested, it is apparent that the delay or suppression of the water-drive response to prolactin given 16 hours after the time of corticosterone injection results from corticosterone and not from handling itself. More complete tests in fish, lizards, and birds also indicate that adrenal steroids and not handling itself entrains daily rhythms of fattening responses and of pigeon cropsac responses to prolactin (Meier, Trobec, Joseph and John, 1971).

DISCUSSION

The finding of a daily variation in prolactin's effectiveness to promote the eft water drive further illustrates the circadian basis of the responses to prolactin. The results indicate also that the circadian variations in the water-drive response to prolactin are synchronized by circadian oscillations of the interrenal system. These conclusions are essentially analogous to those made with respect to the daily variations of fattening (Meier, Trobec, Joseph and John, 1971; Meier and Martin, 1971), locomotor activity (Meier and Martin, 1971) and pigeon cropsac (Meier, Trobec, Joseph and John, 1971; Meier, John and Joseph, 1971) responses to prolactin.

In order for the daily variations in the water-drive response to prolactin to be meaningful, there must also be circadian oscillations of endogenous prolactin and corticosterone. Whether or not these rhythms are present during the life cycle of the newt is unknown. However, circadian rhythms of both hormones have been reported in a variety of vertebrates. Circadian rhythms of adrenal steroids have been particularly well researched (for review, see Halberg, 1969). Circadian rhythms of pituitary prolactin have also been reported in mammals (Clark and Baker, 1964; Kent, Turnbill and Kirby, 1964) and birds (Meier, Burns and Dusseau, 1969).

A possible physiological role for the circadian water-drive response is not readily discernible. It can be argued that although the rhythm of responsiveness may be curious, it cannot be very important. After all, most of the efts did migrate to water eventually regardless of the times of injection. Moreover, prolactin alone induces water drive in hypophysectomized efts (Grant and Grant, 1958; Grant, 1959), suggesting that although other hormones dependent on the pituitary may have auxiliary roles supporting migration to water, they are not necessary for promoting water drive itself.

Upon closer examination, the arguments against important roles for other hormones in controlling water drive do not appear so conclusive. For example, prolactin injections at more than 3 different times during the day with respect to the photoperiod or to corticosterone injections may have uncovered an interval of complete insensitivity to prolactin. A daily interval of complete insensitivity of the pigeon cropsac was found when the number of times of day tested was increased (John and Meier, in preparation; Meier, John, and Joseph, 1971). Similarly, there is an interval during the day when prolactin injections can inhibit completely the progress of tadpole metamorphosis, whereas at other times prolactin is only partially effective or may have no inhibitory effects (Breaux and Meier, 1971).

The fact that prolactin induces water drive in hypophysectomized efts does not indicate necessarily that other hormones dependent on the pituitary are relatively unimportant in regulating water drive. Removal of the pituitary eliminates not only positive factors but also possible negative factors which might act with prolactin to control water drive under natural conditions. Suppositions that a non-migrating eft does not release sufficient amounts of pituitary prolactin is not based on experimental proof. Inhibition of tissue and behavioral responses to prolactin by another factor dependent on the pituitary can just as readily account for the prolonged terrestrial stay (2 to 3 years) of the red eft. Removal of the inhibitory factor could allow prolactin to induce water drive. This study reveals that corticosterone may have such an inhibitory role on the water-drive response to prolactin. However, it is notable that the inhibitory effect of corticosterone on the water-drive response to prolactin occurs 16 hours after corticosterone injection and not at the time of injection. Thus, the simultaneous injection of prolactin and corticosterone (the usual method in testing for augmentation or inhibition) would not reveal the blocking action of corticosterone. In addition, the random injection of prolactin into intact non-hypophysectomized efts is more likely to occur during a time when endogenous corticosterone is not exerting an inhibitory effect on the water-drive response.

The system that could emerge as the regulator of water drive might be one analogous to that described for the regulation of body fat levels in a variety of vertebrates (Meier, Trobec, Joseph and John, 1971; Meier and Martin, 1971). In the White-throated Sparrow, for example, the time of the daily release of pituitary prolactin (Meier, Burns and Dusseau, 1969) and the time of rise in plasma corticosterone (Dusseau and Meier, 1971) change from season to season in a manner that accounts for the seasonal fluctuations in body fat stores. Conceivably, the interval between the daily rhythms of corticosterone and prolactin in the newt may change during the life of the animal resulting in changes in behavioral and physiological states.

The ovine prolactin (NIH-P-S8: 1 mg = 28 I.U.) was a gift of the Endocrinology Study Section of the National Institutes of Health.

SUMMARY

Injections of prolactin late during a 16-hour daily photoperiod are more effective in promoting the red eft water drive than are injections of prolactin given early or at midday. The daily variation in the water drive response to prolactin appears to be phased by the photoperiod and mediated by the interrenal system. The time of corticosterone injection sets the daily rhythm of water drive responsiveness to prolactin. Prolactin is most effective at 8 hours following corticosterone, and least effective about 16 hours after corticosterone.

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THE EFFECT OF TEMPERATURE ON THE ACTIVITY OF BLUEFISH, *POMATOMUS SALTATRIX* L.[1]

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Temperature is one of the most important environmental stimuli exerting an influence on the natural habits of marine organisms. An understanding of the precise role played by this ubiquitous stimulus on the activity of a species is of both fundamental and applied importance. Our aim in this work is to examine, quantitatively, under controlled laboratory conditions, the way in which changes in temperature act upon established behavior patterns in a marine pelagic species. Although there is considerable literature on the responses of fish to temperature (for reviews see Fry, 1964, 1967; de Sylva, 1969; for bibliographies see Kennedy and Mihursky, 1967; Raney and Menzel, 1969; Coutant, 1969, 1970b); there appear to be few published accounts on the subtle changes in behavior of marine pelagic species induced by slowly changing temperature.

Using previously established changes in swimming speed and schooling behavior as our criteria for normal activity of a small group of adult bluefish, *Pomatomus saltatrix* (Olla and Studholme, 1972), we measured the effects of a gradual rise and fall in temperature. Our goal was to predict, with some confidence, the effects of particular thermal levels on natural populations.

MATERIALS AND METHODS

The subjects of our studies were six adult bluefish, 55–65 cm, held in a 121 kl aquarium under conditions of controlled light and temperature (Olla, Marchioni and Katz, 1967). A specialized lighting system simulated natural diurnal changes in light intensity and duplicated natural seasonal photoperiod. Salinity ranged from 23.0–24.5‰ and oxygen from 3.5–6.3 ml/l. Several months after the fish were introduced into the tank, they were in a healthy condition, free of any external signs of infection and feeding regularly.

Since our aim was to observe changes in speed and schooling tendency in free-swimming animals, the number of fish used was important. Based on the space limitations of the tank, preliminary observations indicated that 6–8 adult bluefish constituted a group small enough to avoid crowding yet large enough to be reflective of real changes in speed and grouping.

We conducted the following studies to observe the effects of both a gradual increase and decrease of temperature on swimming speed and schooling tendency. Our speed measurements consisted of five stopwatch readings made every hour of the time for the lead fish of the largest group to swim a measured distance (335 cm). We used the median of these readings for subsequent analysis. We measured the tendency to school by recording, at the same time of the speed readings, the largest number of fish swimming together in the same direction at relatively the same speed within three body lengths. The medians of these counts were used to determine changes in schooling tendency.

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We raised and lowered water temperature by controlling room temperature and water inflow, which resulted in a variation of about ±0.2° C throughout the tank.

We fed the fish live mummichogs, *Fundulus heteroclitus,* 50–125 mm, following the procedures outlined in a previous study (Olla, Katz and Studholme, 1970).

Low temperature

In this experiment we observed the effects of a gradual decrease in temperature on the activity of the fish for 29 days under a natural seasonal photoperiod ranging from 11.16–10.30 hr. The rate of decrease ranged from 0.004–0.042°C/hr (mean 0.012° C) with the exception of 2 days when, due to operational problems, the temperature remained at 14.9° C. Holding temperatures for the fish preceding the experiment were as follows: third month, 22.5 ± 0.5° C; second month, 22.0 ± 0.5° C; first month, 21.0 ± 1.0° C. Then for 18 days prior to the temperature decrease, we held the fish at 19.5 ± 0.5° C and at a light regimen corresponding to the natural seasonal photoperiod during which their rhythmic activity, schooling and feeding showed day-to-day stability. We will refer to this temperature (19.5° C) as the acclimation level (Fry, 1967) for this experiment. At the low temperature limit at which signs of stress were evident (as determined by significant changes in swimming speed and schooling tendency) the temperature remained constant for 24 hours and then increased (mean 0.023° C/hr) until the fish no longer showed signs of stress. Then we lowered the temperature (mean 0.024° C/hr) and when stress became apparent, raised it (mean 0.023° C/hr) toward the acclimation level. Following each 4-day set of observations, while the temperature continued to change, the fish were fed to satiation. Measurements on speed and schooling were suspended during feeding and were resumed 56–60 hr later.

High temperature

In this experiment we observed the effects of a gradual increase in temperature over a 32-day period under a natural seasonal photoperiod ranging from 15.75–16.20 hr. The rate of increase ranged from 0.002–0.038° C/hr (mean 0.021° C. Holding temperatures for the fish preceding the experiment were as follows: third month, 20.0 ± 1.0° C; second month, 20.0 ± 0.6° C; first month, 19.9 ± 0.7° C). Then for 21 days prior to the temperature increase, we held the fish at 19.9 ± 0.5° C and at a light regimen corresponding to the natural seasonal photoperiod. We will refer to this temperature (19.9° C) as the acclimation level for this experiment. At the upper temperature limit as determined by the stress responses of the fish, the temperature was held constant for 6 days and then gradually decreased (mean 0.020° C/hr). Following each 4-day set of observations, we held the temperature constant and fed the fish to satiation. Measurements resumed 29–30 hr later.

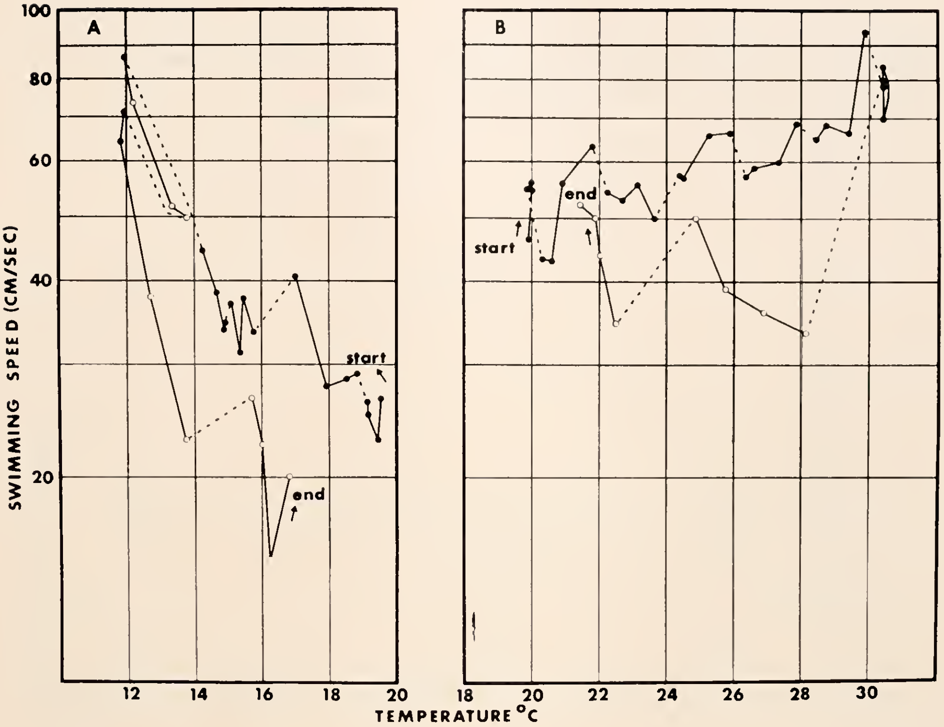

FIGURE 1. (A) Mean daily swimming speeds recorded during low temperature experiment under photoperiods ranging from 11.16–10.30 hr. Black circles represent speeds measured during decreasing temperature; open circles, speeds measured during increasing temperature; dotted lines indicate 72-hr interval. (B) Mean daily swimming speeds recorded during high temperature experiment under photoperiods ranging from 15.75–16.20 hr. Black circles represent speeds measured during increasing temperature; open circles, speeds measured during decreasing temperature; dotted lines indicate 48-hr interval.

RESULTS

Swimming speed

Low temperature: We calculated the mean daily speed by averaging the hourly medians and plotted these values against mean daily temperature (Fig. 1-A). As temperature decreased, mean speed increased until 14.3° C. From this level to 11.9° C, swimming speed nearly doubled. At this low temperature there was also a general lightening in color around the insertion of the pelvic fins. Whether this was due simply to the change in temperature as Abbott (1969) found in *Fundulus heteroclitus*, or like the marked increase in speed was indicative of stress, was not determined. We then raised the temperature 2.3° C over a 3-day period. This rise was accompanied by a rapid decrease in speed approaching that recorded at 14.3° C. Immediately following this we lowered water temperature from 14.2° C to 11.8° C over a 5-day period. This again resulted in a rapid increase in swimming speed.

As the temperature rose smoothly toward the acclimation temperature, swimming speed was depressed below that observed during the initial drop, finally coming to approach the mean speeds recorded at the acclimation temperature. Coloration at the base of the pelvic fins returned to normal.

The coefficient of variation (c.v. $= \sigma'/\bar{x} = \tilde{R}_5/2.257\bar{x}$; Ferrell, 1958) showed

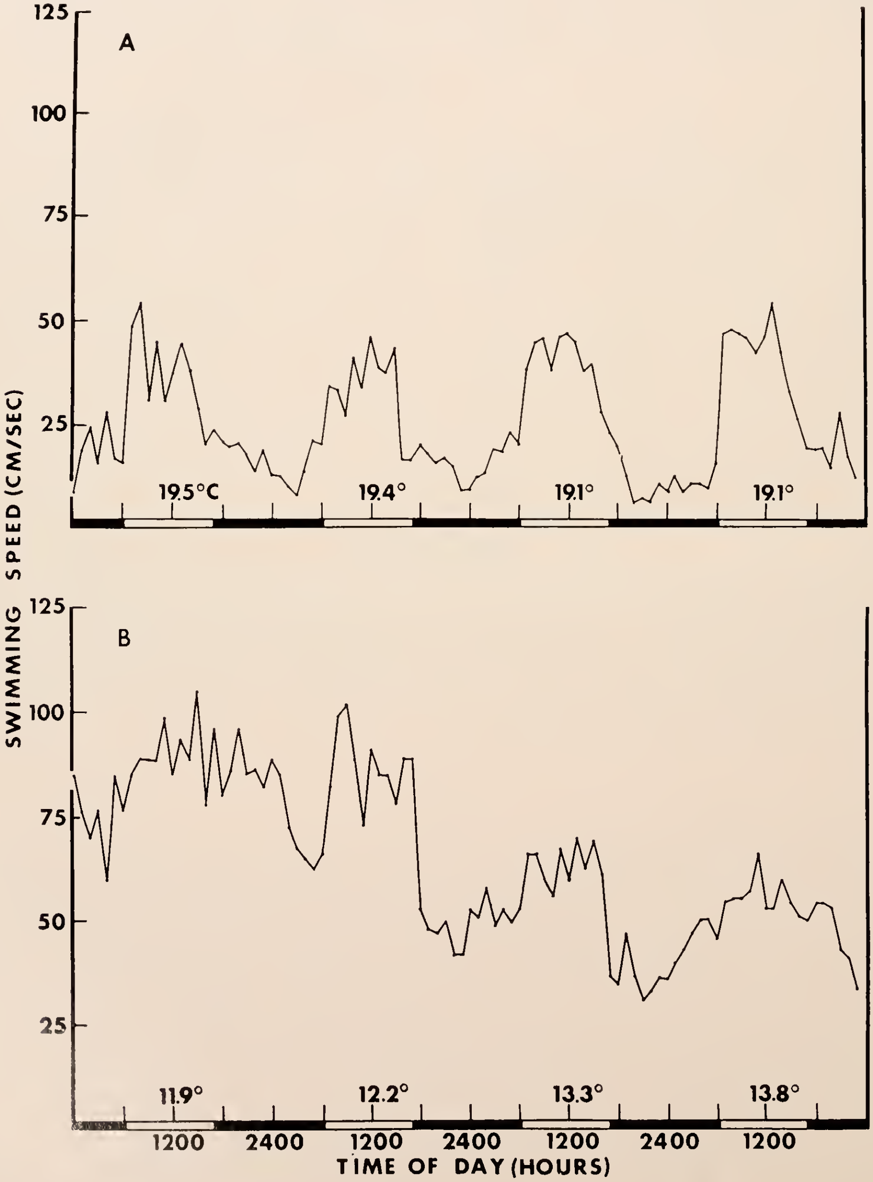

FIGURE 2. Daily swimming speed rhythm measured during low temperature experiment: (A) acclimation, (B and C) stress and gradual recovery, and (D) recovery.

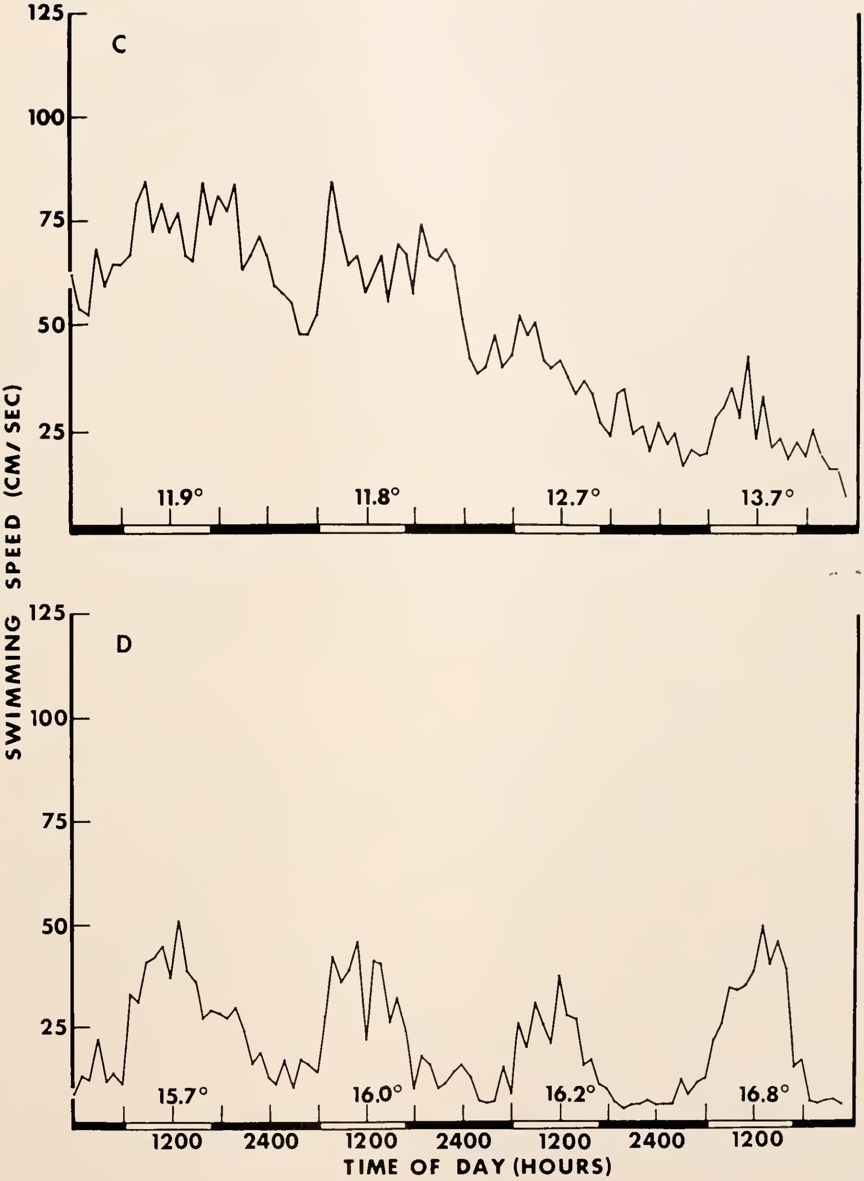

FIGURE 2—*Continued*

that the inherent minute-to-minute variability in swimming rate at the acclimation temperature was higher at night (c.v. = 22.0%) than during the day (c.v. = 13.6%). When the temperature reached 11.9° C, analysis showed that under stress, minute-to-minute variation decreased during both day (c.v. = 9.6%) and night (c.v. = 12.4%), the greater change occurring at night. As the temperature rose to 12.7° C following the second stress period, variation during the day (c.v. = 11.3%) and night (c.v. = 11.2%) were essentially the same.

High temperature: As the temperature increased to 29.3° C, mean speed gradually increased (Fig. 1-B). When the temperature reached 29.8° C, swimming speed increased rapidly within a 24-hr period. Speeds remained relatively high while the temperature was held at 30.4° C. There was also a marked increase in the opening of the mouth and opercula. Similar to findings by Cocking (1957, 1959a) on roach (*Rutilus rutilus*) at stress temperatures, body color above the lateral line darkened. These changes along with the increase in speed we considered to be indicative of stress.

As temperature dropped from 30.4° C to 28.1° C, swimming speed decreased rapidly. As was true following stress at low temperatures, swimming speed was depressed below levels recorded during the period of temperature rise. Near the acclimation temperature, swimming speed approached pre-test levels. Coloration returned to normal as did the gape of the mouth.

At the acclimation temperature of 19.9° C, there was a higher degree of minute-to-minute variability in swimming speed at night (c.v. = 18.9%) than during the day (c.v. = 10.5%). When the temperature reached 29.8° C, variability in speed decreased under dark conditions (c.v. = 13.2%), and increased slightly during the day (c.v. = 14.7%); *i.e.* differences between day and night almost disappeared. In contrast with recovery from low temperature stress, variation increased markedly both day (c.v. = 17.5%) and night (c.v. = 27.8%), well above that observed at 19.9° C.

Daily rhythm of swimming speed

Low temperature: As the temperature decreased and the speed gradually increased, the daily rhythm persisted until the temperature fell below 12.0° C (Fig. 2, A–B). At this point the fish swam at significantly greater speeds at night with consequent diminution of the rhythmic pattern although there were still significant differences between day and night ($P < 0.05$; Tukey-Duckworth End Count Test, Tukey, 1959). As the temperature rose above 12.0° C, speeds decreased and a well-defined daily rhythm was evident. As the temperature dropped below 12.0° C for a second time (Fig. 2-C), the fish resumed swimming at high speeds both day and night and for a period of about 41 hours, there was no significant difference between day and night ($P > 0.05$). Unlike the previous temperature recovery period, there was still no significant separation of day and night speeds ($P > 0.05$) until the temperature went above 12.7° C (Fig. 2, C–D).

High temperature: As the temperature rose and the mean speed increased, the daily rhythm persisted until the temperature reached 30.4° C (Fig. 3, A–B). Then as we had observed at low temperature stress levels, there was no significant difference betwen day and night speeds for 48 hours ($P > 0.05$; Tukey-Duckworth End Count Test). Although the mean daily temperature remained at 30.4° C for the two following days, significant day-night differences reappeared ($P < 0.01$). As the temperature dropped below 30.4° C and swimming speeds dropped sharply, there was no significant day-night difference for 24 hours ($P > 0.05$). Then throughout the rest of the temperature recovery, *i.e.*, as the temperature dropped below 28.1° C, a daily rhythm was evident (Fig. 3-C).

Table I

Day-night schooling tendency as related to changing temperature

Low temperature							High temperature						
Avg. temp. °C	Schooling index*		Δ = D-N	III compared to			Avg. temp. °C	Schooling index*		Δ = D-N	III compared to		
	Night	Day		I	II	IV		Night	Day		I	II	IV
19.5	2.5	4.4	+1.9	+			19.9	2.7	4.8	+2.1			
19.4	1.8	3.4	+1.6	+			20.0	1.5	5.9	+4.4	+		
19.1	1.5	4.3	+2.8	+			20.0	2.6	5.9	+3.3	+		
I 19.1	2.2	5.4	+3.2	+			19.8	2.1	5.7	+3.6	+		
18.8	3.5	5.5	+2.0	+			I 20.3	2.5	5.7	+3.2	+		
18.5	2.2	4.1	+1.9	+			20.6	2.1	5.5	+3.4	+		
17.9	2.3	5.0	+2.7	+			20.9	4.3	5.8	+1.5			
17.0	4.1	5.9	+1.8	+			21.8	2.4	5.6	+3.2	+		
							22.3	1.7	5.5	+3.8	+		
15.7	3.9	5.4	+1.5		+		22.7	1.7	5.6	+3.9	+		
15.4	3.4	4.4	+1.0		+								
15.3	2.7	5.9	+3.2		+		23.1	2.9	5.5	+2.6		+	
II 15.1	3.3	4.9	+1.6		+		23.6	2.2	5.8	+3.6		+	
14.9	4.3	5.5	+1.2		+		24.4	3.0	5.5	+2.5		+	
14.9	2.5	4.8	+2.3		+		24.5	3.1	5.9	+2.8		+	
14.7	3.4	5.7	+2.3		+		II 25.2	2.5	5.8	+3.3		+	
14.3	4.6	5.9	+1.3		+		25.8	2.9	5.8	+2.9		+	
							26.4	4.3	6.0	+1.7			
11.9	5.8	6.0	+0.2	−	−	−	26.6	4.0	5.9	+1.9			
12.2	6.0	5.6	−0.4	−	−	−	27.3	2.1	5.7	+3.6		+	
III 13.3	5.5	5.9	+0.4	−	−	−	27.8	2.3	5.1	+2.8		+	
13.8	5.3	5.9	+0.6	−	−	−							
11.9	5.7	5.8	+0.1	−	−	−	28.4	5.2	6.0	+0.8	−	−	
11.8	5.9	5.7	−0.2	−	−	−	28.7	5.2	5.7	+0.5	−	−	
							29.3	5.3	5.8	+0.5	−	−	
12.7	3.7	5.5	+1.8			+	III 29.8	5.7	6.0	+0.3	−	−	−
13.7	2.3	3.7	+1.4			+	30.4	3.8	6.0	+2.2			
IV 15.7	2.2	4.9	+2.7			+	30.4	5.0	5.0	0	−	−	−
16.0	2.5	5.5	+3.0			+	30.4	5.5	5.8	+0.3	−	−	−
16.2	1.5	4.8	+3.3			+	30.4	5.2	6.0	+0.8	−	−	
16.8	1.5	5.5	+4.0			+							
							28.1	3.0	5.6	+2.6			+
							26.9	3.1	5.7	+2.6			+
							25.7	2.1	4.4	+2.3			+
							IV 24.9	1.9	5.4	+3.5			+
							22.5	1.4	4.5	+3.1			+
							22.0	2.5	5.0	+2.5			+
							21.9	3.1	3.9	+0.8			
							21.4	3.2	3.7	+0.5			
Sign test			26+/ 2−							35+/ 0−			
End Count test				8 6	8 6	6 6					8 7	8 7	6 3
$P \leq$			0.01	0.001	0.001	0.01				0.01	0.001	0.001	0.01

* Schooling index = $\sum$(freq. 6 fish × 6 + freq. 5 fish × 5 . . . freq. 1 fish × 1)/100 where frequency = % of occurrence of each group size measured 5 times each hour.

Schooling

Low temperature: Similar to our findings under normal conditions (Olla and Studholme, 1972), the tendency to school throughout the experiment was significantly greater during the day than at night ($P < 0.01$; Sign Test; Table I). At

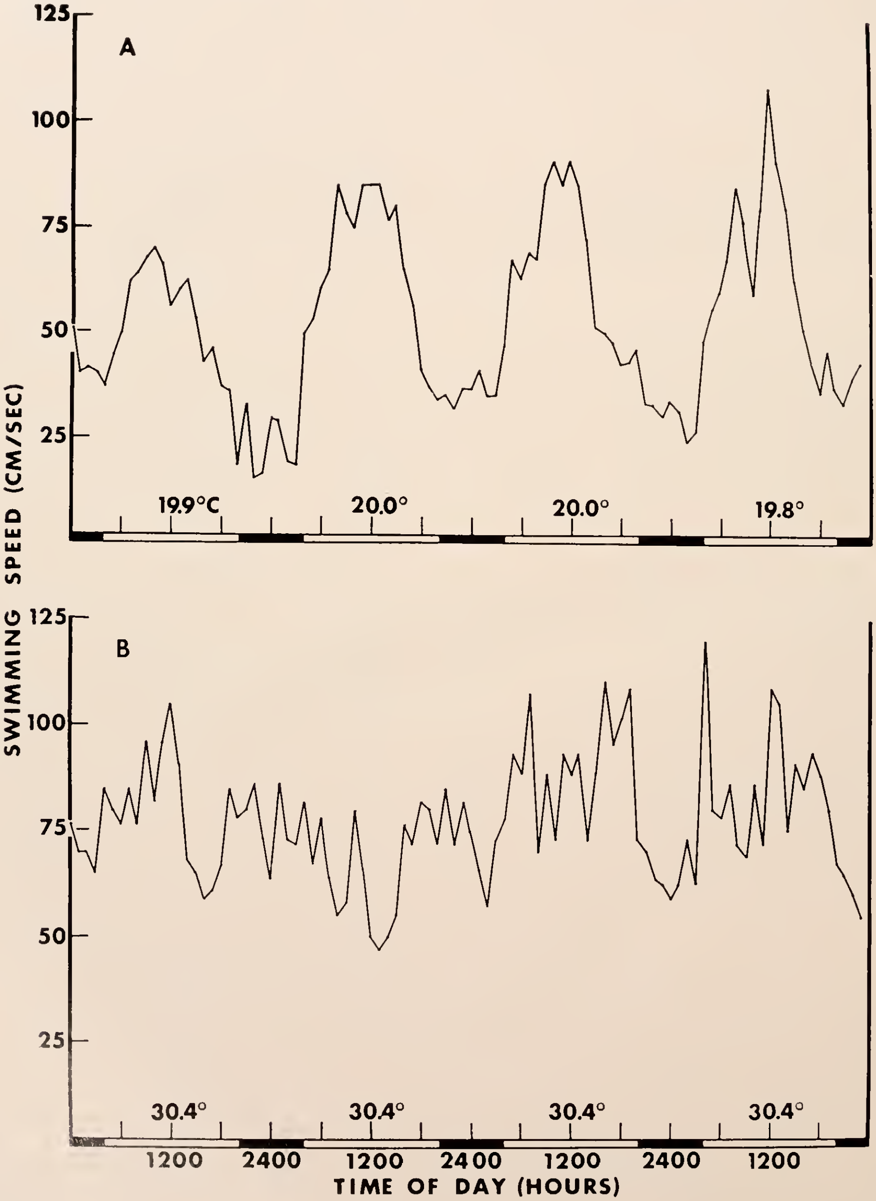

FIGURE 3. Daily swimming speed rhythm measured during high temperature experiment: (A) acclimation, (B) stress, and (C) recovery.

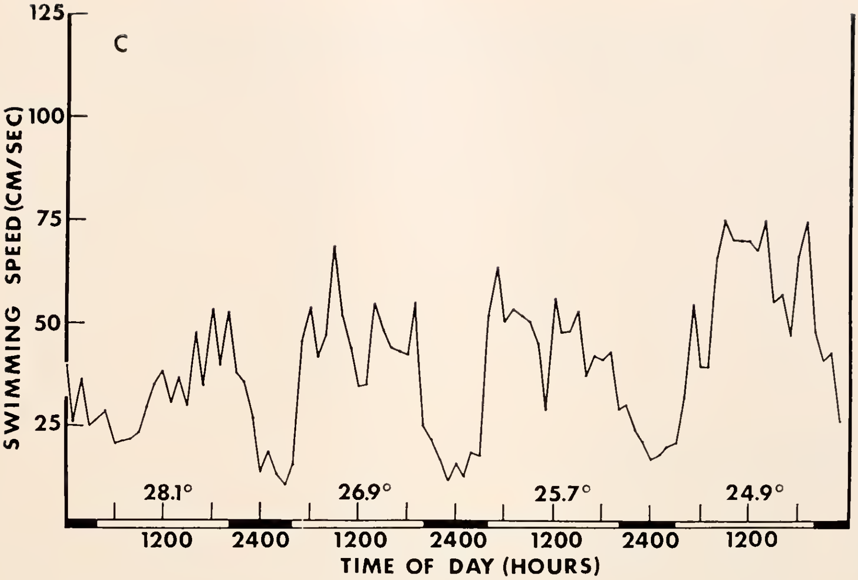

FIGURE 3—*Continued*

a temperature of 11.9° C, night schooling increased. The difference in the tendency to school between day and night was significantly reduced at low stress temperatures ($P < 0.01$; Tukey-Duckworth End Count Test).

High temperature: Throughout the experiment, schooling tendency was again significantly greater during the day than at night ($P < 0.01$; Sign Test; Table I). When the temperature reached 28.4° C, schooling tendency increased significantly at night, reducing day-night differeneces ($P < 0.01$; Tukey-Duckworth End Count Test). Towards the end of the temperature recovery, small day-night differences reappeared.

Feeding

Low temperature: Feeding, as measured by the quantity of food ingested, was relatively stable until the temperature dropped to 13.7° C at which point there was a 40% decrease compared with amounts taken during acclimation (Table II). Feeding remained low following stress temperatures and then increased slightly as the temperature rose.

High temperature: Feeding fluctuated considerably as the temperature increased to 30.2° C (Table II). Following the 6-day period of constant temperature of 30.4° C, feeding decreased and showed no appreciable increase as the temperature returned toward acclimation levels.

DISCUSSION

The most discernible response to both high and low temperature stress was the increase in swimming speed by about $3\frac{1}{2}$ times above acclimation speeds at low temperature stress and $1\frac{1}{2}$ times above acclimation speeds at high temperature

TABLE II

Weight of live prey ingested as related to temperature changes

Low Temperature*		High Temperature**	
Avg. temp. °C	Grams taken	Avg. temp. °C	Grams taken
19.0	2180	20.0	2399
16.6	2070	22.2	3136
15.0	2210	24.2	1973
13.7	1310	26.1	2586
14.1	1470	28.3	2324
15.1	1810	30.2	1963
16.9	1750	30.4	1442
		24.1	1440
		21.2	1513

* Feeding intervals 7 days.
** Feeding intervals 6 days.

stress. The speed increase with increasing temperature probably was less than that at decreasing temperature because the initial average speed was high due to the seasonal effect of longer photoperiods (Olla and Studholme, 1972). Since this seasonal effect would tend to complicate a comparison of the rate of change in swimming speed, it is possible that had both experiments been conducted under the same photoperiod the rate of increase might have been similar. However, average speeds at both stress levels were of the same magnitude, *i.e.*, about 70–80 cm/sec. It is likely that the lower speeds observed at acclimation presumably are normal for bluefish at that temperature and at those photoperiods.

The decrease in well-defined patterns of rhythmicity at stress temperatures was due to a decrease in the difference between day and night speeds. The peak-to-trough differences diminished as a result of proportionately greater increases in night speeds. Other indications of response to stress were also clearly evident at night. Variation in speed, usually high at night, was significantly less. Schooling tendency, normally low, showed a significant increase, approaching daytime levels. Whether the increase in schooling tendency at night at thermal stress levels is the result of the increase in swimming speed or is due to a direct change in fish-to-fish responsiveness could not be determined.

In contrast to the similarity in the responses of the fish to both cold and hot temperature stress were several differences observed when the temperature was returning to acclimation levels. Following low temperatures, the day-night schooling pattern returned to normal; following high temperatures, after an initial return to normal schooling patterns for about 10 days, there was a sharp decrease in day-night differences matching those observed under stress. Feeding gradually increased during temperature recovery from low stress but remained relatively low for at least two feeding sessions following high temperatures.

Depressed speeds, abnormal schooling tendency and feeding following exposure to high temperatures may indicate, that while the external source of stress has been removed, the animal has in effect not really returned to normal. This could be the result of a delay in acclimation related to the exposure to stress. Fish subjected to high temperatures and then returned to non-stress conditions may be more susceptible to predation (Coutant, 1970a), disease (Cairns, 1956), and less able to function as successful predators. Cairns (1956) found that though feeding in bluegill (*Lepomis macrochirus*), pinfish (*Lagodon rhomboides*) and channel catfish (*Ictalurus punctatus*) increased, the fish became emaciated following prolonged exposure to "sub-lethal" temperatures. Our results showed no consistent change in feeding during the experiment except following stress temperatures when prey capture decreased.

Peterson and Anderson (1969), in their work on Atlantic salmon (*Salmo salar*), found that regardless of the direction of temperature change, locomotor activity increased. Their experiments showed that it was the rate (from about 0.1–0.7° C/min) rather than just the amount of change which influenced the increase in activity. In our own work, the slow rate of temperature change would probably negate any strong influence of rate on change in activity. Although Cocking (1959b) states that a rate of 1/20° C/hr temperature increase was sufficient to permit continual acclimation in roach, it is our feeling that there may be a delay in the acclimation of the bluefish to both the increase and decrease in temperature. In current work on Atlantic mackerel (*Scomber scombrus*), swimming activity continued to increase for several time constants although the temperature increase (0.5° C/day) was stopped well below lethal levels. This would indicate that although the rate of increase was low, a delay existed in the acclimation of the animals (Olla, in preparation). However, in our findings on bluefish, presumably any delay is slight and constant so that the observed responses, at least at the upper levels, can be related to specific temperatures.

Thermal limits established here are only relevant to the particular size range under study. We would expect, as has been noted for other species, that differences in temperature responses would be dependent on size and age (de Sylva, 1969).

Since seasonal changes in photoperiod affect thermal lethal limits (Hoar and Robertson, 1959; Tyler, 1966; Graham, 1970), it is essential that these results be considered relative to photoperiod. Additionally, since these limits were based on the responses of slowly acclimated animals, animals subjected to rapid temperature changes in the environment, due to natural or unnatural causes, would respond at different thermal levels, dependent on the acclimation temperature and season.

There are basic differences in the responses of pelagic and benthic fishes to temperature extremes. For example, puffer (*Sphaeroides maculatus*) are highly unresponsive at night, normally lying quiescent on the bottom. Wicklund (1970) states that a massive kill of puffer which occurred during an abnormal temperature drop at night may have been due to this low level of responsiveness. In field studies on another benthic species, winter flounder (*Pseudopleuronectes americanus*), Olla, Wicklund and Wilk (1969) found that these fish, normally day-active, became inactive as the temperature rose above 22.3° C. Laboratory studies on summer flounder (*Paralichthys dentatus*) showed that when subjected to temperature drops averaging 3° C/hr, this semi-benthic fish became quiescent with a drop in the cardiorespiratory rate of more than 50% (Olla and Wicklund, unpublished). In contrast, some pelagic species such as the bluefish and many of the scombrids, must swim continually to (1) maintain hydrostatic equilibrium due to an insufficient or absent swim bladder (Magnuson, 1970); (2) ventilate the gills (Hall, 1930; Magnuson, 1963); or (3) aid in venous circulation through continued contraction of skeletal musculature. These fish react to stress temperatures with increased responsiveness. The nature of the response to both low and high temperature extremes may serve to move the animals out of areas of adverse temperature. Lund and Maltezos (1970) state that when the water temperature falls below 15° C, adult bluefish begin their fall migration. This relates to our findings of generally high speeds at temperatures below 15° C.

We wish to express our grateful appreciation to Enoch B. Ferrell for his advice on the statistical treatment of the data.

SUMMARY

1. The swimming speed of the bluefish increased as temperature increased or decreased from acclimation levels of 19–20° C.

2. As the temperature approached 11.9° C and 29.8° C, there were significant changes in average swimming speed and schooling which were considered to be indicative of stress.

3. The daily rhythmic activity was not well-defined at stress temperatures.

4. As the temperature departed from stress levels toward acclimation, swimming speed dropped significantly and the daily rhythm of activity returned.

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ROLE OF SYMBIOTIC ALGAE (ZOOXANTHELLAE) IN CORAL CALCIFICATION

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Members of many invertebrate groups live symbiotically with unicellular algae, but the symbiosis between corals and dinoflagellate algae (zooxanthellae) is especially interesting because it occurs in all species of tropical reef-building corals (see reviews by Droop, 1963; Yonge, 1963; McLaughlin and Zahl, 1966). Moreover, a significant effect of the algae on the physiology of corals has been clearly demonstrated and quantified: Corals with symbiotic algae calcify many times faster in light than in darkness, while corals which have lost their zooxanthellae calcify at rates which are slower and unaffected by light (Kawaguti and Sakumoto, 1948; Goreau, 1959; Goreau and Goreau, 1959). In the light, photosynthesis by zooxanthellae must somehow lead to higher rates of calcification by corals.

Three mechanisms have been proposed to explain how zooxanthellae influence coral calcification: (1) removal of carbon dioxide in photosynthesis directly favors chemical equilibria leading to the precipitation of calcium carbonate (Goreau, 1959); (2) algal removal of phosphates, which may act as crystal poisons, enhances crystallization of calcium carbonate (Simkiss, 1964a, 1964b); and (3) organic products of photosynthesis, either specific materials required for skeletogenesis, or nutrients or general energy sources supplied to the coral, permit faster calcification (Goreau, 1959; Wainwright, 1963). So far, there has been no experimental evidence which conclusively supports or eliminates one hypothesis or another.

One observation appears to be inconsistent with current ideas about the intimate relationship between algal photosynthesis and coral calcification. In the staghorn coral, *Acropora cervicornis* (Fig. 1), as in other branching forms, calcification rates are highest in the tips, decreasing progressively towards the base (Goreau and Goreau, 1959). However, very few symbiotic algae are found in the tips, their numbers increasing towards the base. Where abundant, they give the coral a deep brown color, contrasting sharply with the whiteness of the almost algae-free tips (Figs. 1 and 2). We undertook a study of the rapidly calcifying tips in order to clarify the problem of how the algae stimulate coral calcification rates.

MATERIALS AND METHODS

Collection and incubation

Corals were collected from shallow reefs (1–3 meters) in Discovery Bay on the north coast of Jamaica and kept in running seawater for up to a few hours until the start of each experiment. All light experiments were run outdoors in natural light (500–2000 footcandles), but not in direct sunlight. Dark controls were run simultaneously in blackened boxes. In a few experiments, the tips of some of the coral branches were covered with opaque cylindrical caps, 10 mm long, 7 mm in diameter, covered with aluminum foil and lined with black plastic tape

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(Fig. 3). Temperature in both light and dark was maintained at 26° ± 1° C. Coral branches cut off 30 mm from the tip were incubated in seawater to which radioisotope had been added, in shallow, transparent glass or plastic vessels. The vessels contained about 80 mg coral/ml seawater and were stirred occasionally during incubation. At the end of incubation, the corals were washed in fresh seawater to remove unused radiosotope, and sections of standard sizes were cut for processing. Several sections were sometimes pooled and counted together. Incubations lasted 2–4½ hours.

Labeling with calcium-45

To measure calcification, coral branches were incubated in seawater with 1–2 μc/ml $Ca^{45}Cl_2$. Sections were dissolved in hydrochloric acid, and aliquots of acid were plated on planchets, dried, and immediately assayed for radioactivity with a Nuclear Supplies Model SA 250 scaler and GM Lionel Anton 1007T thin end-window tube.

Labeling with carbon-14

To measure accumulation of organic carbon, coral branches were incubated in seawater with 1–3 μc/ml $NaHC^{14}O_3$. The sections were processed for organic carbon-14 determinations by two different methods. In some experiments, the samples were placed in hot 3 *N* KOH to remove the tissue, which was then homogenized in a glass tissue grinder and brought to known volume; aliquots were plated, dried, and assayed for radioactivity as above. In others, the corals were extracted several times with warm 80% ethanol, then with a mixture of absolute methanol and chloroform (2:1); these were combined as a "soluble" tissue fraction and acidified before counting. HCl was added to the insoluble residue to decalcify the skeleton, and the resulting tissue suspension was homogenized. Aliquots of both soluble and insoluble fractions were plated, dried, and counted as above.

Both calcification and accumulation of organic carbon are expressed as counts per minute (Ca^{45} or C^{14}) per microgram protein nitrogen in the sample, corrected for background and self-absorption. It should be noted that the values do not necessarily represent constant rates, but rather the total amount of calcium-45 or organic carbon-14 accumulated in a given period. Thus, absolute values can only be compared among controls within a given experiment, for which all experimental conditions were alike. Incubation time, light intensity and temperature probably constitute the most important variables among conditions in the different experiments.

Determination of protein nitrogen

Protein nitrogen values were determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Commercially standardized bovine serum albumin was used as a standard. Pieces of coral were heated in concentrated ammonium hydroxide for about one hour. The tissue was then brought into suspension by gentle agitation, and the clean skeleton was removed. The tissue suspension was homogenized in a glass tissue grinder and brought to known volume; aliquots of this homogenate were taken for protein nitrogen determinations. Since it was technically difficult to determine both radioactivity and protein nitrogen from the same

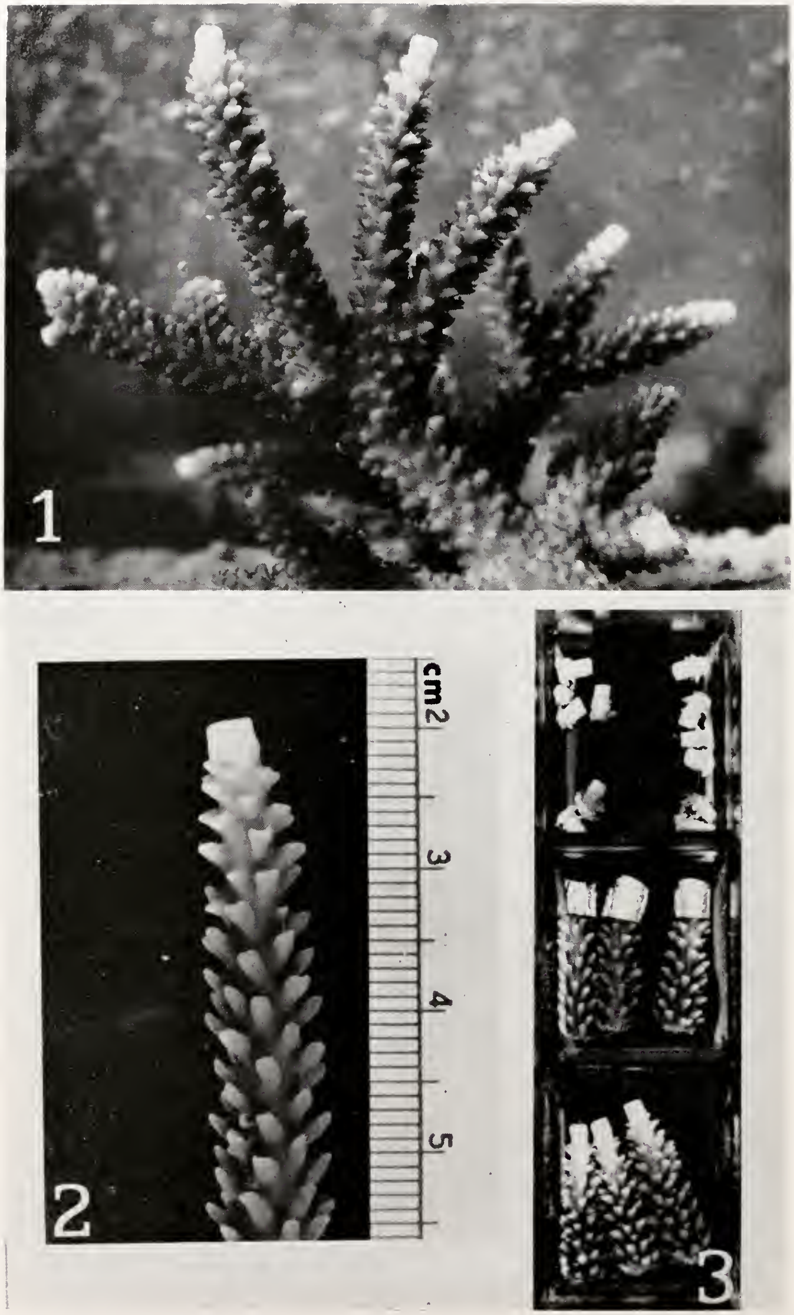

FIGURE 1. Small colony of the reef-building coral *Acropora cervicornis.* Each branch bears many lateral polyps and a single large terminal polyp. As the branch grows outward, new lateral polyps continually develop in a zone just below the terminal polyp and others are added at lower levels as the diameter of the branch increases. The deep brown color of the

experimental sample, protein nitrogen values were determined for a number of non-radioactive samples of standard sizes, and average protein nitrogen values were used in calculations.

Determination of chlorophyll

The relative amounts of zooxanthella chlorophyll in coral tissue were determined by spectrophotometric readings of pigment extractions, after the method of Richards with Thompson (1952; see also Strickland and Parsons, 1965). Standard sections of coral from three different regions of the branches were cut and placed in test tubes, 5 sections of each region per tube. To each tube, 3.0 ml 90% acetone and a drop of an aqueous suspension of $MgCO_3$ were added. The tubes were filled with nitrogen, tightly stoppered, and stored in a dark refrigerator for 24 hours, with occasional stirring. The final extracts were centrifuged, and optical density (uncorrected values, determined directly from the extracts) was read in a Bausch and Lomb Spectronic 20 spectrophotometer at 630 nm for chlorophyll *c* and 665 nm for chlorophyll *a* against a blank containing 90% acetone.

Chromatographic procedures

Two-dimensional radiochromatography and identification of labeled unknowns were carried out as described previously (Muscatine, 1965; Muscatine and Cernichiari, 1969), following the procedure of Benson, Bassham, Calvin, Goodale, Haas and Stepka (1950). The procedure for deacylation of lipids is also described by Muscatine and Cernichiari (1969).

RESULTS

Gradients in coral branches

In the first series of experiments, chlorophyll, protein nitrogen, dry weight (mostly calcium carbonate), and calcification in light and dark (as calcium-45 labeling) were measured in three successive sections of coral branches from the tip downwards (Fig. 4). The term "tip" designates the terminal polyp only. In calcification experiments, intact 30 mm coral branches were incubated in seawater with added calcium-45 in light and dark, and sections of the branches were cut at the end of the incubation period.

Figure 4 shows two features which are immediately apparent by simply examining a coral branch (see also Goreau, 1963). First, the amount of algal pigment in the branch increases from the tip towards the base (Fig. 4A). Although the zooxanthellae were never counted, tissue smears revealed a gradient in the numbers of algal cells which paralleled the data for chlorophyll. Second, the dry weight per protein nitrogen of each section (tissue plus skeleton) increases from the tip towards the base (Fig. 4B), as the tissues constitute a greater proportion

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tissue is due to large numbers of symbiotic zooxanthellae; the white tips contain very few zooxanthellae.

FIGURE 2. A single branch, enlarged to show the details of algal distribution and developing polyps near the tip.

FIGURE 3. Incubation chambers containing (*top*) 5-mm pieces bearing "isolated" tips, (*center*) branches with opaque caps covering the tips to shield them from light, (*bottom*) 30-mm branches with intact tips.

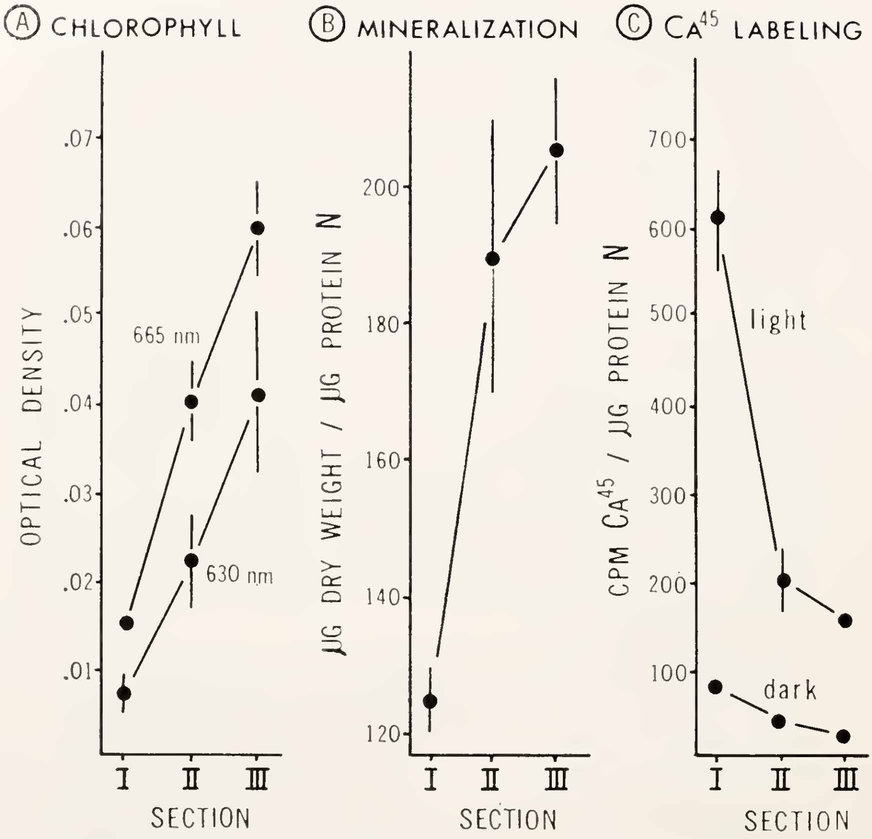

FIGURE 4. Gradients in coral branches. (Mean values ± one standard deviation). *Section I:* 0–3 mm, terminal polyp only, lightly calcified, few zooxanthellae. *Section II:* 3–6 mm, developing lateral polyps, well calcified, more zooxanthellae. *Section III:* 6–9 mm, fully-developed lateral polyps, well calcified, abundant zooxanthellae.

of the total weight in the soft, lightly calcified tip than in the heavily calcified basal portions. This suggests that calcification should take place most rapidly in the tip, the rate decreasing basally, as has indeed been found by Goreau and Goreau (1959) and as our calcium-45 data confirm (Fig. 4C). The curves in Figures 4B and 4C are thus approximately reciprocal.

The intrinsic gradient in calcification rates, decreasing from the tip downwards, is expressed by the dark values in Figure 4C. We expected that in the light, this gradient would be tempered or even reversed, since the enhancement of calcification rates by light would be greatest in the basal portion of the branch, where zooxanthellae are most abundant, while the white tips would be least affected. Surprisingly, however, we found the reverse: The intrinsic calcification gradient was actually reinforced in the light, and the effect of light on calcification, reflected in the light/dark ratios calculated for each section (Fig. 6, solid curve), was consistently greatest in the tip (Section I), where zooxanthellae were rare. The effect

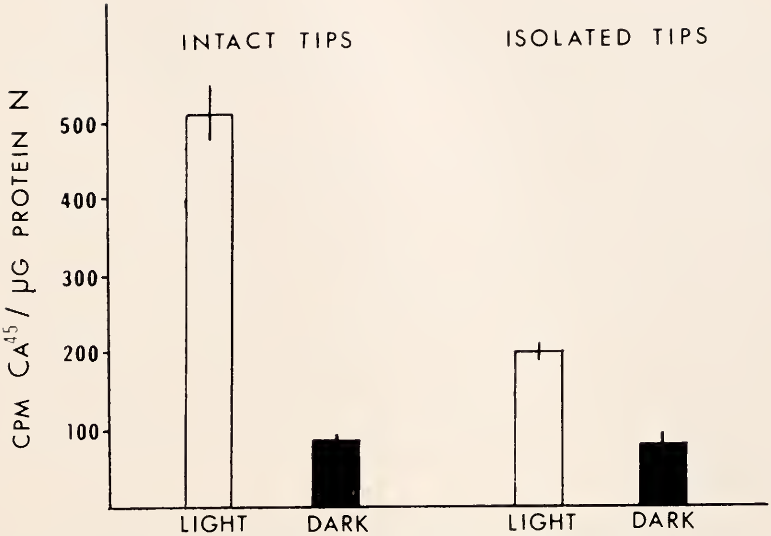

FIGURE 5. Calcification of intact and isolated tips, in light and dark. (Mean values ± one standard deviation, each representing 9 coral tips pooled in 3 lots of 3 tips each.)

of light was consistently least in Section II, with intermediate numbers of zooxanthellae. Light/dark values varied in different experiments, but this pattern was constant.

Measurements of calcification

To explain the high light/dark ratios for calcification in the algae-poor tip, we hypothesized that algae in lower portions of the branch might be enhancing calcification rates in the tip. To test this possibility, we ran a second series of calcification experiments in which some 30 mm branches were incubated with the tips intact as before, while in other branches, the terminal 5 mm (*i.e.*, the tip plus a portion of Section II) was cut off and incubated alone. A brown zone of zooxanthellae was usually visible in the bases of these 5 mm pieces (see Figs. 2 and 3), so the tips were not completely isolated from adjacent algae but were cut off from the bulk of the algae in the branch. All were incubated with calcium-45 in light and dark. After incubation, only the 3 mm tip (terminal polyp, Section I) was cut from each piece for calcium-45 determination. The results of one of these experiments are presented in Figure 5.

In the dark, intact and isolated tips appeared to calcify equally. The dark controls thus serve also as controls for possible damage or other effects of isolating the tips. In the light, calcification in intact tips increased six fold, while calcification in isolated tips increased only two fold over dark levels.

To determine the effect of darkening the tips only, another calcification experiment was run in which the tips of some of the coral branches were covered with opaque caps (see Methods and Fig. 3). In this experiment, summarized in the first half of Table I, intact and isolated tips show the same relationships to dark controls as before. Calcification in intact, capped tips on illuminated branches was greater than in tips of branches which were wholly dark, consistent with the hypothesis of translocation. Dark controls with and without caps indicated that the caps themselves had no apparent effect on calcification. Isolated tips placed inside caps in the light calcified at the same levels as dark controls, confirming that the caps did effectively exclude light.

To resolve the paradox of finding the highest light/dark ratios in the algae-poor tips, Sections II and III from branches with intact tips were also analyzed in this experiment. The light/dark ratios of Sections I, II and III (Fig. 6, solid line) followed the usual pattern, with the tip showing the highest ratio and Section II, the lowest. However, if the light and "dark" (capped) values for isolated tips (Table I) are substituted (Fig. 6, dotted line), the tip then has the lowest light/dark ratio. The ratios increase towards the base, paralleling the increase in abundance of algae (Fig. 4A), as would be expected. The seemingly great effect of light on tip calcification in the almost complete absence of algae must therefore depend on algae present in lower portions of the branch. When the tip is experimentally isolated from the bulk of the algae, the effect of light on calcification is much diminished.

The results of our calcification experiments seemed to indicate, in summary, that: (1) There is a gradient in calcification rates from the tip downwards, in both light and dark (Fig. 4C); (2) intact tips show the greatest light enhancement of calcification (Fig. 6, solid curve), although they contain fewer zooxanthellae than lower portions (Fig. 4A), but (3) if values for isolated tips are used, the light enhancement gradient parallels the algal gradient, as would be expected (Fig. 6, dotted curve). (4) Intact tips calcify faster than isolated tips in the light, but not in the dark (Fig. 5); and (5) light enhancement of calcification is still seen when the tip itself is dark and only lower portions of the branch are illuminated (Table I).

These results all support the hypothesis that rates of calcification in the coral tips were stimulated in some way during photosynthesis by zooxanthellae in lower portions of the branch. Since transfer of materials from zooxanthellae to the tissue

TABLE I

Calcification and organic carbon in intact and isolated coral tips; effect of darkening tips only. (Each Ca^{45} value represents 3 tips pooled together. Each C^{14} value represents 6 tips pooled together; C^{14}-labeled tissue was fractionated by extraction with alcohol-chloroform mixtures)

	cpm Ca^{45}/μg protein N	cpm C^{14}/μg protein N		
		Soluble	Insoluble	Total
Intact tips, in light	160, 160	23.8	12.1	35.9
Isolated tips, in light	79, 80	16.9	6.7	23.6
Intact tips, in light, with caps	85, 93	5.2	2.9	8.1
Intact tips, in dark	51, 54	2.0	1.7	3.7
Intact tips, in dark, with caps	52, 57			
Isolated tips in light, with caps	52, 57			

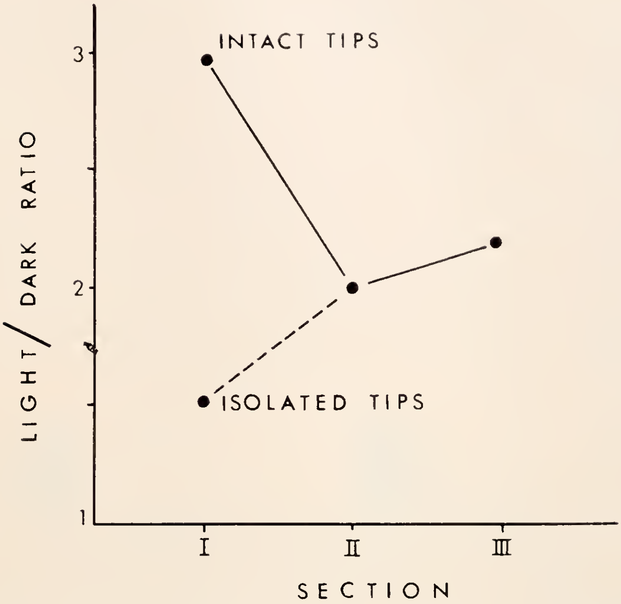

FIGURE 6. Light/dark ratios for calcification: intact vs. isolated tips. (Sections as in Fig. 4.)

of a coral had been demonstrated previously (Muscatine and Cernichiari, 1969), we hypothesized further that calcification might be stimulated through an organic photosynthetic product, translocated to the tip.

Measurements of accumulation of organic carbon

To test the possibility that photosynthetically fixed organic carbon was translocated from the algae to the coral tip, 30 mm branches with intact tips and isolated tips on 5 mm pieces (Fig. 3) were incubated in seawater containing added carbon-14 as sodium bicarbonate, in light and dark. After incubation the 3 mm tips only were removed for assay, just as in the calcium experiments above. The results of one of the carbon-14 experiments in which the tissue was removed in KOH (see Methods) are presented in Figure 7. Dark values probably represent primarily heterotrophic fixation by coral tissue, and were not significantly different in intact and isolated tips. In the light, however, intact tips contained significantly more organic carbon-14 than isolated ones.

In another experiment, summarized in the second half of Table I, the tissue components were fractionated by extraction with alcohol-chloroform mixtures (see Methods). As it was necessary to pool the samples, only single values were obtained and the level of significance of the differences observed is uncertain. However, the results were uniformly consistent with those from whole tissue. Intact tips contained more organic carbon-14 than isolated ones, and capped tips with illuminated bases contained more organic carbon-14 than dark controls. These differences appeared in both soluble and insoluble fractions.

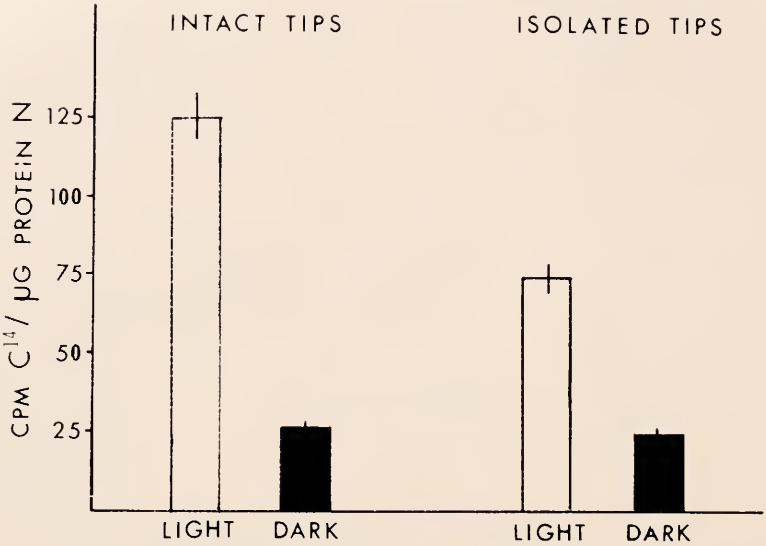

FIGURE 7. Organic carbon-14 in intact and isolated tips, in light and dark; whole tissue in KOH. (Mean values ± one standard deviation, each representing 9 coral tips pooled in 3 lots of 3 tips each.)

It is likely that our values from 5-mm "isolated" tips consistently overestimate both calcification and accumulation of organic carbon in the tip proper, because (1) the few algae in the tips themselves are included in our C^{14}-accumulation data, although their contained products are not strictly within the coral tissue, and (2) the bases of the "isolated" tips include the first developing lateral polyps with substantial numbers of zooxanthellae, the tip proper being too soft and fragile to incubate alone, and although these algae are not included in the final assay, their products may be translocated to the tip during incubation (compare light and dark isolated controls, Fig. 7). Values for capped tips, on the other hand, probably underestimate calcification and organic carbon accumulation (Table I) in a darkened tip, because the 10-mm caps, necessary to shade the tip completely, also partially shade adjacent lateral polyps with their contained zooxanthellae. Thus the differences between intact and isolated tips are consistently minimized by the experimental conditions, and the differences between uncapped and capped tips are exaggerated.

In addition, although the caps appeared to have no direct effect on calcification (compare capped and uncapped dark controls, Table I), capped branches released more than three times as much fixed organic carbon-14 into the seawater medium as did the uncapped branches. In two experiments in which the seawater medium was assayed, the total fixed carbon-14 in tips + medium was approximately the same in both capped and uncapped controls, but uncapped branches released only 18% and 21% of the total into the medium while capped branches released 54% and 53%. Neither the mechanism nor the significance of this release to the medium is understood. Perhaps increased release resulted from cell damage by the caps, but this seems unlikely, as the caps fitted very loosely (see Fig. 3) and, as noted above, appeared to have no direct effect on calcification (Table I). It is possible also that darkening of the tip inhibits axial translocation and leads instead to release of translocated material to the medium; or that translocated products accumulate in the confined medium inside the cap and reach concentrations which inhibit further translocation, again leading to increased release. The explanation of this phenomenon awaits further investigation, but it is probably responsible for the fact that capped tips accumulate less organic carbon than one might expect even from the minimal differences measured between intact and isolated tips. Thus although capped tips compare unfavorably with free, intact ones in the light, capped tips with illuminated bases do exceed tips of dark controls in both calcification and accumution of organic carbon (Table I), consistent with the hypothesis that axial translocation of organic photosynthetic products somehow stimulates calcification rates in the tip.

Analysis of C^{14}-labeled products

In an effort to localize the label into as few products as possible, a "pulse-chase" experiment was also run. Coral branches were incubated in the light for 30 minutes in seawater containing 3 μc/ml $NaHC^{14}O_3$. All tips were intact, and all were covered with opaque caps in order to minimize photosynthesis by the few algae in the tips. At the end of the "pulse," the branches were quickly washed in fresh seawater, and a zero-time sample of tips was processed immediately to yield soluble and insoluble fractions (see Methods). Tips were isolated (cut off at 5 mm) from half of the remaining branches; the rest were left intact. Both groups were placed in the dark in fresh seawater without radioisotope for 3 hours and then processed as usual into soluble and insoluble fractions. The values obtained (cpm organic $C^{14}/\mu g$ protein N, each value representing 6 tips pooled together) from tips at zero-time and from intact and isolated tips at 3 hours were, respectively, as follows: Soluble: 4.8, 5.9, 3.6; Insoluble: 2.7, 2.9, 2.6; and Total: 7.5, 8.8, 6.2.

In the intact tips, carbon-14 activity appeared to increase with respect to the zero-time sample, suggesting that some labeled product or products, photosynthetically fixed by the algae in lower portions of the branches during the light incubation, continued to move distally into the intact tips during the dark period. The isolated tips showed a loss of labeled carbon, compared to the zero-time sample, probably due primarily to respiration over the 3-hour dark period. The difference between intact and isolated tips appeared most evident in the alcohol-chloroform soluble fraction. Soluble material from intact, isolated, and intact capped tips, incubated with $NaHC^{14}O_3$ in the light, was therefore analyzed by two-dimensional paper chromatography in order to identify the labeled product or products. The results are given in Table II.

By far the most carbon-14 was contained in lipids, followed by glycerol and glucose. This large percentage of label in lipids suggested the likelihood of glycerol translocation since this compound is known to be released *in vivo* (Muscatine and Cernichiari, 1969), followed by synthesis of lipids in the coral tissue. Deacylation showed that lipids were indeed labeled only in the glycerol moiety.

Intact, isolated, and intact capped tips were incubated in anticipation that their labeled contents would include different proportions of the translocated materials.

TABLE II

Per cent distribution of organic C^{14}-labeled products in the soluble fraction of coral tissue. (Each value represents 12 coral tips pooled together)

Compound	Intact tips	Isolated tips	Intact tips, capped
glucose	6.78	6.48	5.15
glutamine	2.21	1.13	2.94
alanine	0.81	0.45	0.76
glycerol	8.64	7.88	6.04
lipids	80.18	83.32	81.17
unknown	0.53	1.31	3.60

However, there appeared to be no qualitative differences in carbon-14 distribution among them, suggesting that translocation from algae adjacent to the tips, even in isolated tips as discussed above, may be a much greater source of labeled carbon than algae within the tips themselves.

DISCUSSION

The results of our calcium-45 experiments suggest that calcification in the tip of a branch of *Acropora cervicornis* is increased in the light as a result of photosynthesis by symbiotic algae farther down in the branch. If so, the mechanism by which the zooxanthellae stimulate calcification must be one which can act over some distance. We put forward for testing the hypothesis that some organic product or products of algal photosynthesis, translocated axially to the coral tissue, in original or altered form, stimulate calcification, especially in the tip.

Our carbon-14 experiments indicate that axial translocation of algal products does take place in *Acropora*. It has been shown (Muscatine and Cernichiari, 1969) that, in the coral *Pocillopora damicornis*, 35–50% of the total photosynthetic product is excreted by the zooxanthellae, primarily as glycerol. In the coral tissue, the glycerol is converted largely to lipids, and the skeletal organic matrix has a substantial lipid component, consisting mostly of cetyl palmitate (Young, 1969; S. D. Young, J. D. O'Connor and L. Muscatine, in preparation). In *Acropora*, Lewis and Smith (1971) found that glycerol, glucose and alanine appear to be translocated *in vivo* by the zooxanthellae, and we also found glycerol (free and in lipid) and glucose to be the major labeled products in the coral tips (Table II). The chemistry of the skeletal organic matrix of *Acropora* has not been studied, and we did not attempt to recover it for separate analysis. However, if it is similar to those in other corals, the algal products could provide the raw materials for several major components: the glycerol could be incorporated into lipids; the glucose, into N-acetylglucosamine (see also Wainwright, 1963); and the alanine, into protein. All of these could also be used less specifically as general sources of energy for skeletogenesis.

We suggest that translocated algal products may enhance calcification rates in corals by serving either as specific substrates in the organic matrix or as general energy sources. The fact that calcification and translocation are diminished approximately to the same extent when the *Acropora* tip is isolated from the bulk of the zooxanthellae in the branch (Figs. 5 and 7) provides support for this hypothesis. But there is still no direct evidence for the ability of algal products to stimulate calcification in any coral.

Goreau (1959) found that even in the dark, normal corals with zooxanthellae sometimes calcified 2 to 3 times faster than corals which had lost their zooxanthellae. He suggested that translocation of organic products from zooxanthellae may have been responsible for this dark enhancement of calcification. Since our pulse-labeling experiment indicated that some translocation of organic products to *Acropora* tips did continue in the dark, one might expect small differences between intact and isolated tips in the dark as well as in the light. Simkiss (1964a, 1964b) also suggested that zooxanthellae might stimulate calcification in the dark by absorbing phosphates which are potential inhibitors of calcification. Yamazato (1966) found that phosphate uptake by the coral *Fungia scutaria* did continue in the dark, although dark uptake rates were only 14% of those in the light. We found no significant differences in calcification between intact and isolated tips in the dark; if either translocation or phosphate uptake is in fact related to calcification, the quantities involved were insufficient to show measurable differences in the dark under our experimental conditions.

Translocation may also account for another seeming paradox in *Acropora* data. Goreau (1963) calculated approximately equal productivity values for terminal and lateral polyps of *Acropora* on the basis of organic carbon-14 measurements. The relatively high values for terminal polyps were unexpected in view of their much lower chlorophyll content and fewer zooxanthellae, and Goreau proposed an explanation in terms of shading effects. We suggest the alternative possibility that his values for terminal polyps may have included a large component of translocated organic carbon.

Goreau (1963) also compared calcification in corals and coralline algae, particularly in relation to productivity. Recent studies have demonstrated (Pearse, in preparation) several striking parallels between the characteristics of coral calcification discussed here and calcification in coralline algae of the genus *Bossiella* and probably *Amphiroa:* (1) the terminal portion of each branch of a plant is less heavily calcified than more basal portions; (2) there is a decreasing gradient in calcification rates from the terminal to more basal portions, in both light and dark; (3) calcification rates are greater in light than in darkness (see also Goreau, 1963); (4) the increase in calcification rates in the light is greatest in the terminal portion; and (5) the terminal portion appears less heavily pigmented, often nearly white, compared with more basal portions. Although there is no information about translocation in these plants yet, our studies on corals suggest that it is a likely possibility.

The hypothesis that zooxanthellae enhance coral calcification rates through translocation of organic carbon produced in photosynthesis in no way excludes the carbon dioxide hypothesis of Goreau or the phosphate hypothesis of Simkiss. Any or all of these mechanisms may operate in many corals. However, since the mechanisms proposed by Goreau and Simkiss both depend on the maintenance of a strong concentration gradient of carbon dioxide and phosphate respectively, they seem less likely to be effective where the site of calcification is at some distance from the bulk of the zooxanthellae, as in the rapidly calcifying tips of branches of *Acropora.*

We dedicate this paper to the late T. F. Goreau[2] in gratitude for the stimulating leads he provided in his extensive work with corals and for making available to us the facilities of the UWI-SUNY Marine Laboratory in Discovery Bay, Jamaica. We thank Mrs. E. Cernichiari and Mr. N. Copland for technical and administrative assistance; Mr. R. Pool for help in collecting corals; Mr. H. Reiswig and Mrs. S. Sidman for assistance with figures; Drs. D. C. Smith and D. H. Lewis for many helpful suggestions during the course of this work; and Dr. J. S. Pearse for critical reading of the manuscript. Financial support was provided by a postdoctoral fellowship from the National Institutes of Health (to V. B. P.) and National Science Foundation grant GB-3728 (to L. M.).

SUMMARY

1. In branches of the coral *Acropora cervicornis*, the abundance of symbiotic algae (zooxanthellae) increases from tip to base, while active calcification decreases. Light enhancement of calcification rates is, paradoxically, greatest in the algae-poor tips of branches.

2. Calcium-45 experiments on intact and isolated tips of the coral branches suggest that light enhancement of calcification in the algae-poor tip results from photosynthesis by zooxanthellae farther down in the branch.

3. Carbon-14 experiments indicate that organic products of algal photosynthesis are translocated to the coral tip. The main carbon-14 labeled products in the tip are lipids, glycerol and glucose.

4. Our data are consistent with the hypothesis that translocated algal products enhance coral calcification rates.

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EFFECTS OF SALINITY AND STARVATION ON RELEASE OF DISSOLVED FREE AMINO ACIDS BY *DUGESIA DOROTOCEPHALA* AND *BDELLOURA CANDIDA* [PLATYHELMINTHES, TURBELLARIA][1]

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The tissues of marine invertebrates contain very high levels of free amino acids (FAA) which are believed to serve an intracellular osmoregulatory function (*e.g.*, Florkin and Schoeffeniels, 1965). Potts (1967) suggested that as a consequence of these high FAA levels and the tendency of these compounds to leak across the body wall into the water, marine invertebrates probably lose more FAA to the water than do comparable freshwater invertebrates. Consistent with this hypothesis was our observation that both tissue FAA levels and FAA release rates of the marine turbellarian, *Bdelloura candida* increased with increasing salinity (Johannes, Coward and Webb, 1969).

Relative *respiratory* costs of coping with marine *versus* freshwater environments have been calculated (Potts, 1954; Potts and Parry, 1964). If differences in FAA release rates between marine and freshwater invertebrates exist as a consequence of differences in tissue FAA levels, this implies another type of differential energy loss related to osmoregulation—one in which studies of the respiratory energy costs of osmoregulation do not take into account. Here we compare FAA release rates of *Bdelloura* and its freshwater relative (*Dugesia dorotocephala*), on an energetic basis.

In addition, we use our data to demonstrate how some differences of opinion in the literature concerning FAA release rates by marine invertebrates can perhaps be reconciled if the relationships between release rates and the rate of feeding and time since feeding are taken into consideration.

METHODS AND MATERIALS

The freshwater planarian *Dugesia dorotocephala* used in these experiments were from a permanently sexual strain originally collected in Oklahoma and maintained in the laboratory on a diet of chicken liver. Specimens of *Dugesia* were kept in a dilute artificial saline medium (Coward, 1968) for both maintenance and experimental situations. All procedures, including aseptic conditions, involving *Dugesia dorotocephala* and *Bdelloura candida* were as previously described (Johannes, Coward and Webb, 1969), with appropriate use of the dilute saline (0.35‰) for *Dugesia* rather than seawater: FAA samples of 2.5 hour duration were taken at precise 24 hour intervals with time zero being the cessation of feeding; the time zero incubation sample contained some presumably egested material and mucus (which were removed by the routine Millipore filtration step) but the subsequent samples did not. FAA samples were analyzed as previously described (Johannes,

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Coward and Webb, 1969). The bound amino acids from the 80% ethanol insoluble residues of chicken liver were determined after 20 hours of 6 N HCl hydrolysis at 100° C.

Values for caloric content of most amino acids ($-\Delta H^0_c$, K cal/mole) were obtained from Hutchens (1970). The value for taurine (382.9) was obtained from Kharasch (1929). The values for proline (649) and histidine (747) were determined by bomb calorimetry and the values for ornithine (722) and lysine (879) were calculated by the method of Kharasch (1929).

Respiration of *Bdelloura* was measured in a 25 ml respirometer equipped with Ag-Pt electrodes and a paddle type stirrer operating at about 120 rpm. The continuously recorded signals, representing the oxygen concentrations, were linear during the one hour determination at 25° C. Calibration was by Winkler oxygen determination.

Results

Respiration, FAA release rates and FAA in tissues of *Dugesia* after periods of starvation are presented in Table I. Respiration rates dropped 50% over a five-day period, whereas FAA release rates dropped by almost three orders of magnitude.

Comparison of caloric equivalence of released FAA and of respiration for *Dugesia* and *Bdelloura* is presented in Table II. Caloric cost of FAA loss is positively correlated with salinity for *Bdelloura* and was higher at all salinities than the caloric cost of FAA loss in the freshwater *Dugesia.* Weight specific respiration rates were similar for the two species.

The major constituent FAA of both the tissues and release products of *Bdelloura*, and their caloric values are reported in Table III in order of increasing caloric content of the amino acid. A number of FAA individually ≤ 1% of the total have been omitted from both Tables III and IV. The relatively calorically poor amino acid glycine, makes up a much greater proportion of the released FAA than of the tissue FAA. This difference in proportions of FAA between the tissues and release products reinforces our confidence that the animals are not being damaged during handling with a concomitant non-specific release of FAA (Corner and Cowey, 1968).

Table I

Effect of starvation on caloric equivalence of respiration and free amino acid (FAA) release by Dugesia. *Dashes indicate values not determined*

Hours starved	Respiration* (cal × 10^{-3}/g wet wt/hr)	FAA released (cal × 10^{-3}/g wet wt/hr)	FAA loss caloric (equivalent as % respiration)	Tissue FAA (μM/g wet wt)
0	2250	967.0	43.00	28.3
24	1600	9.3	0.58	13.5
48	1400	3.79	0.27	—
72	1350	3.05	0.23	—
96	—	—	—	9.2
120	1250	1.52	0.12	—

* calculated from Hyman (1919).

TABLE II

Caloric equivalence of respiration and free amino acid (FAA) released by Dugesia *and* Bdelloura *after 24 hours starvation*

	Respiration* (cal $\times 10^{-3}$/g wet wt/hr)	FAA released (cal $\times 10^{-3}$/g wet wt/hr)	FAA loss caloric (equivalent as % respiration)	Tissue FAA (μM/g wet wt)
Dugesia				
0.35‰ salinity	1600*	9.3	0.58	13.5
Bdelloura				
12‰ salinity	1620	12.5	0.77	4.2
19‰ salinity	2350**	18.1	0.77	11.6
26‰ salinity	1610	24.3	1.51	11.6
33‰ salinity	1710	23.0	1.35	32.0

* Calculated from Hyman (1919).
** Animals were extremely active.

The *Bdelloura* tissue FAA and FAA release values are reported as means (Table III) of determinations made at four salinities (Johannes, Coward and Webb, 1969); mole percentages of the various FAA were essentially the same at different salinities (12, 19, 26 and 33‰), unlike those for some other marine invertebrates that have been examined in this connection (*e.g.*, Virkar and Webb, 1970).

Relative proportions of FAA in *Dugesia* tissue and release products after starvation are reported in Table IV. The FAA and bound amino acid content of chicken liver upon which both *Dugesia* and *Bdelloura* were fed are included in Table III. Immediately after feeding, the relative proportions of most of the FAA in the release products (Table IV) and the food (Table III) are similar for *Dugesia*. Proportions in the tissues change with starvation, but do not approximate those of the release products.

TABLE III

Comparison of mole per cent of free amino acids (FAA) in food, tissues and release products of Bdelloura. *Animals starved 24 hours; ± one standard deviation in parenthesis; constituents $\leq 1\%$ omitted*

	$-\Delta H°c$ (Kcal./mole)	Food		*Bdelloura*	
		FAA	Bound amino acid*	Tissue	Released
Glycine	230.5	8.7	8.3	1.7 (0.33)	52 (11.0)
Serine	347.7	7.9	5.1	1.9 (0.24)	7 (2.0)
Taurine	383	11.5	0	0.91 (0.18)	0
Aspartic acid	382.6	7.1	8.4	4.6 (2.0)	3.1 (0.78)
Alanine	387.1	8.8	7.0	18.1 (3.3)	6.7 (1.2)
Threonine	409.7	5.4	4.5	2.8 (1.2)	2.7 (0.37)
Glutamine acid	536.4	13.3	9.3	4.0 (1.1)	3.4 (0.79)
Ornithine	722	0.4	6.9	0.1	2.8 (1.2)
Arginine	893.5	2.6	3.9	7.4 (1.3)	5.1 (1.2)

* Bound FAA are 6 N HCl hydrolysates of 80% ethanol insouble material.

TABLE IV

Changes in relative molar per cent of free amino acids in Dugesia *tissues and release products with starvation. Constituents* ≤ *1% omitted*

	Tissue amino acids			Released amino acids				
	Days starved							
	0	1	4	0	1	2	3	5
Glycine	2.8	1.8	2.4	8.8	15	9.9	12.0	20.8
Serine	5.2	5.1	6.5	2.9	23	20.4	17.8	29.5
Taurine	3.7	5.6	1.3	4.5	16	11.9	13.5	0
Aspartic acid	4.5	9.6	12.9	4.6	6.7	7.1	10.4	11.4
Alanine	9.6	7.3	13.8	11.4	10.9	7.9	trace	trace
Threonine	4.1	2.7	4.1	6.6	5.9	4.5	8.0	0
Glutamic acid	9.2	11.5	15.1	13.2	8.6	10.8	11.0	23.5
Ornithine	1.1	1.7	2.1	0.75	2.7	9.6	8.3	14.8
Arginine	2.3	6.1	5.6	4.0	7.0	trace	0	0

DISCUSSION

Corner, Cowey and Marshall (1965) have shown that ninhydrin positive nitrogen-containing compounds "excreted" by *Calanus* after feeding on *Cricosphaera* declines by nearly an order of magnitude during the first day after feeding. The rate of "excretion" was also related to the level of food input. In the present work, the FAA release rates of *Dugesia* (Table I) dropped by two orders of magnitude during the first day of starvation and by almost another order of magnitude during the next four days. Thus, it appears that the rate of feeding and the elapsed time since eating greatly influence rates of "excretion." These considerations, as well as others (Johannes and Webb, 1970), render the prediction of release rates of compounds by small animals in natural environments difficult. Their recognition, however, may help reconcile some of the controversies in the literature concerning this subject. Corner and Newell (1967) stated that they could not find any evidence for the release of FAA by *Calanus helgolandicus* in either starved or "feeding" animals. Obviously, an animal must have at least as much nitrogen available in the food as it is releasing, otherwise it will become nitrogen starved. The animals used by Corner and Newell either were starved for three or more days after capture or fed at a level which supplied only 23% of their measured nitrogen release. Consequently, their results do not approximate release rates under natural situations.

In Corner and Newell's (1967) criticism of our work (Johannes and Webb, 1965) they correctly report that using our equation, the predicted rate of release of FAA for *C. helgolandicus* at 10° C would be 4 μg alpha-amino N/mg dry body weight/day, or approximately the rate they observed for total nitrogen release from starving animals. Our equation was derived from values obtained on newly captured and presumably unstarved animals. It would thus be more appropriate to compare our predicted value of 4 μg with values obtained for total nitrogen release of actively feeding animals, which are probably within the range of 10–20 μg (Corner, Cowey and Marshall, 1965).

Little and Gupta (1969) imply that the peak FAA release in turbellarians should occur at 24 hours after feeding on the basis that the blind guts would be voided of food remnants approximately 24 hours after feeding (Jennings, 1957, page 67). The suggestion has been made by Little and Gupta (1969) that the release data for *Bdelloura* previously published (Johannes, Coward and Webb, 1969), and in part included here, was misinterpreted because the measurements were made after 24 hours of starvation and thus presumably during egestion. Virtually all visible egesta were voided during the first few hours of starvation by our experimental animals. In addition, maximum release rates of FAA appear to occur at the end of the feeding period in *Dugesia* (Table I) and decline progressively with time of starvation.

The order of magnitude drop of FAA release by *Dugesia* between day 0 and day 1 (Table I) obviously has major ecological implications as well as a direct bearing on the design of experiments to measure release products. It may also bear a relationship to the mechanism(s) involved in the release. Odum (1961) has suggested that the release of ingested radioisotope occurs in two phases, the first consisting of a rapid loss of non-assimilated material, and the second, a slower loss of assimilated or waste material, the loss rate being related to food consumption as well as temperature, growth, and reproduction. In analogous fashion, if the FAA released immediately after feeding are primarily non-assimilated material, the relative proportions of FAA released should be similar to those of the food, as modified by selective absorption. Such appears to be the case, since after taking into account the ratio of 18:1 for bound: FAA in the food, two of the three amino acids (glycine and glutamic acid) most frequent in the release products are also found to be most frequent in the food.

A priori, it seems reasonable that the source of released FAA after 5 days of starvation would be the tissue derived FAA. The data of Table IV indicates that such a loss must be quite selective. Alanine, a major tissue FAA, is a minor component of the release products after five days of starvation. However, it is more predominant in the release products after feeding than it is in the food.

Our present data (Table II) plus unpublished data from these laboratories on FAA release by other marine and freshwater invertebrates supports the hypothesis of Potts (p. 33, 1967) that "Marine invertebrates might be expected to lose more amino acids than freshwater ones." Regression analysis of the salinity—FAA release data (Table II) gives a correlation coefficient (r) of 0.95 for the 5 salinities.

Unlike the escape of unassimilated FAA from the gut, loss of FAA by excretion and leakage represents a true loss of metabolic energy by the animal. It ap- that this loss is greater in the marine turbellarian *Bdelloura* than in its freshwater relative *Dugesia* and increases with increasing salinity (Table II). This extra energetic cost associated with living in a marine environment is on the order of 1% of the energy loss associated with respiration in *Bdelloura,* and is of the same order as the extra respiratory energy calculated to be expended by freshwater invertebrates in coping with their hypoosmotic environment (Potts, 1954). Thus it appears that the extra respiratory cost of osmoregulation in the hypoosmotic freshwater medium may be more or less balanced energetically by the extra cost, via FAA leakage, of maintaining the high intracellular FAA levels associated with invertebrates living in seawater. Clearly more work must be done before the relative costs of coping osmotically can be accurately known for seawater *versus* freshwater environments. There may be additional factors complicating this relationship, for example, Sharma (1968) reports that energy loss via urea production by *Orconectes rusticus* increases proportionally to the ambient salinity.

The ubiquitousness of glycine and serine as the major components of samples analyzed for FAA seems worthy of discussion. They are very abundant in human sweat (Oro' and Skewes, 1965, Hamilton, 1965), human urine (Hamilton, 1970), release products of zooplankton (Webb and Johannes, 1967), release products of other aquatic invertebrates (Johannes and Webb, 1970) and in the free form in fresh water, estuaries and seawater (Brehm, 1967; Hobbie, Crawford and Webb, 1968; Bohling, 1970). Glycine is the most abundant amino acid in so-called prebiotic experiments (Fox and Windsor, 1970) and from extraterrestrial sources (Kvenvolden, Lawless, Pering, Peterson, Flores, Ponnamperuma, Kaplan and Moore, 1970). This, perhaps, all seems reasonable because glycine is structurally the simplest amino acid and contains the least amount of covalently bound energy. In the present data glycine and serine make up from 12–60% of the total released FAA (Table III), while they account for only 4–10% of the tissue FAA and only about 14% of the total amino acid in the food used for *Dugesia* and *Bdelloura*. Thus it appears that many species have a relatively low ability to retain these two small molecules of relatively low caloric content. In general, glycine and serine are directly biologically interchangeable; many metabolic pathways terminate in glycine and both glycine and serine are commonly precursors for many syntheses. This seems very reasonable if present biotic systems evolved from abiotic environments with relatively high glycine concentrations and if small molecules served as precursors to an array of larger molecular species. Since glycine is structurally small, calorically poor, and metabolically ubiquitous, it is not surprising that it is both highly available and highly expendable with minimal possible adverse affects on the animal.

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SUMMARY

1. Immediately after feeding, *Dugesia* loses almost half as many calories in FAA release as via respiration. After one day, calorie loss via FAA release drops to less than 0.6% of that lost through respiration.

2. Loss of FAA from 24 hr starved animals of *Dugesia* and *Bdelloura* is highly correlated with environmental salinity ($r = 0.95$).

3. FAA loss appears to occur in two phases, the first consisting of a rapid loss of non-assimilated material, and the second a lesser and gradual loss of (presumably) assimilated material.

4. Glycine or serine, the two lowest energy content amino acids, are predominant among the FAA released by *Bdelloura* and *Dugesia*, respectively.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1971

Abstracts in both sections are arranged alphabetically by author. Author and subject references will also be found in the regular volume index, appearing in the December issue.

ABSTRACTS OF SEMINAR PAPERS

August 10 and 17, 1971

Concentrative accumulation of prostaglandins by some tissues of marine invertebrates and vertebrates. Laszlo Z. Bito, David Turansky and Alice Van Voris.

Tissue pieces not exceeding 100 mg each were isolated from dogfish, *Mustelus canis;* skate, *Raia erinacea;* eel, *Anguilla rostrata;* flounder, *Pseudopleuronectes americanus;* sea bass, *Centropristes striatus;* sea robin, *Priontus evolans;* and the invertebrates spider crab, *Libinia emarginata;* squid, *Loligo pealii;* clam, *Spisula solidissima;* scallops, *Plactopecten magellanicus* and *Aequipecten irradians;* and sea urchin, *Arbacia punctulata.* Isolated tissues or whole animals were incubated in appropriate Ringer solutions or filtered sea water containing tritium labeled prostaglandins (^{3}H-PGs) A_1, E_1, $F_{1\alpha}$ or $F_{2\alpha}$.

Following 90 min of incubation at 21° C with ^{3}H-PGF$_{2\alpha}$ concentrative accumulation of ^{3}H, as measured by the tissue to medium ratio (T/M) was evident by the choroid plexuses, iris, kidney, and the liver or hepatic caeca ($T/M > 1$). No such accumulation was evident in the spleen or gut of vertebrates; in fact, PGs appear to be partially excluded by these tissues ($T/M < 0.8$). The gills, with the exception of those of bivalves, do not accumulate, and probably exclude, PGs. In bivalves, especially in scallops, there is large concentration of ^{3}H activity by the gills, when either the excised tissue or the whole animal is incubated with ^{3}H-PGs. ^{3}H accumulation is temperature dependent, and can be blocked by the addition of large concentrations of an unlabeled PG. The accumulated ^{3}H activity can, at least in part, be eluted from the tissues. The largest concentrative uptake was, in general, observed with ^{3}H-PGA$_1$ (T/M as great as 200).

These results show that a great variety of marine forms possess mechanisms which can interact with prostaglandins, and suggest that PGs, and/or chemically related compounds, may be present and have physiological functions in all marine forms studied.

Supported by USPHS Research Grant EY00333 and EY00402.

Iso-electric gel focusing of maturing duck hemoglobins. Thomas A. Borgese and Richard Egnor.

The technique of iso-electric focusing (IEF) permits the separation of proteins which differ by as little as 0.02 pH units with respect to their iso-electric points. We have previously reported that three hemoglobins were present in the duckling (Hbs I, II, and III) and only two in the adult (Hbs I and II); the most cathodal component in the duckling having disappeared by the 10th to 12th week of post-hatching development. Re-investigation of the hemoglobin pattern in the maturing duck using IEF in polyacrylamide gels in short term studies, confirms the basic patterns for the duckling and adult which were observed with conventional electrophoretic methods. In 72 hour experiments, also employing a disc electrophoresis apparatus, IEF in a pH 7–10 gradient, 50 volts and a current of 1 mAmp per tube, at least three additional bands are observed. IEF of chromatographically isolated Hbs I and II indicate that at least one extra band is derived from the former and two from the latter. In a time course experiment the density of these additional bands seems to increase (by visual inspection) at the expense of the original hemoglobins. Hemoglobin III was not seen in samples of CO-hemoglobin from the adult duck containing as much as 1200 μg per tube, but was consistently seen in duckling samples when as little as 200 μg of hemoglobin was used. Measurements of the approximate iso-electric points of the bands corresponding to Hbs I, II, and III were made after removal from the gel and elution with distilled water following electrophoresis for 72 hours. The values were 7.90, 7.14, and 8.36, respectively. We presently subscribe to the hypothesis that Hb III represents a distinguishing feature of duckling hemoglobin, and that the additional bands seen on prolonged electrophoresis represent post-synthetic modifications of the original hemoglobins.

Supported by grants from the Nutrition Foundation, The Research Foundation-CUNY, and The George Shuster Fellowship Fund of Lehman College.

Spermatophore formation in the vas deferens of the spider crabs. GERTRUDE W. HINSCH AND MURIEL H. WALKER.

The reproductive tract of male specimens of *Libinia* were fixed in a paraformaldehyde-glutaraldehyde mixture, postfixed in OsO_4 and embedded in Spurr or Araldite. Mature sperm pass from testis to vas deferens by way of the seminiferous duct. The sperm become organized into spermatophores of varying size in the anterior vas deferens. Seminal fluid secretion and storage of spermatophores occurs in middle and posterior vas deferens.

The epithelial cells of the anterior region are tall columnar. The basal regions have numerous pliacae surrounding mitochondria. Nuclei are lobulated. Cisternae of rough endoplasmic reticulum run along the length of the cell and are filled with a flocculent material. Numerous, large Golgi complexes are scattered throughout the cytoplasm. Large vesicles containing electron dense secretion products appear in the apical cytoplasm. Microvilli cover the cell surface and project into the secretion product which surrounds sperm and forms spermatophores.

Cells of the mid vas deferens are cuboidal and resemble the above except they lack mitochondria—pliacae association in basal areas. Large "lakes" appear in the intercellular spaces and between septate desmosomes.

Posterior vas deferens cells are columnar and have quantities of vesicular rough endoplasmic reticulum filled with flocculent material. Cell surfaces are covered with microvilli. Seminal fluid in luminal areas is electron dense. Fully formed spermatophores are found in the fluids of the mid- and posterior vas deferens.

Work supported by a grant (F-20-UM-6A) from the Florida Cancer Society.

Dr. Hinsch is an NIH Career Development Awardee.

Mammalian sperm hyaluronidase, an isoantigen of possible interest for fertility control. CHARLES B. METZ, ALBERTO C. SEIGUER AND AMALIA E. CASTRO.

Rabbit semen, but not rabbit seminal plasma, rapidly disperses the cumulus mass surrounding newly ovulated rabbit eggs. Following exposure to antisemen antibodies (goat or guinea pig origin) rabbit semen and sperm extracts fail to disperse the cumulus. Likewise rabbit sperm extracts fail to depolymerize hyaluronic acid in the presence of antisemen antibody. Since univalent (Fab) antibody fragments prepared by papain digestion also produce these effects, antibody must inhibit by a rather direct blocking of hyaluronidase, not by secondary action (*e.g.*, sperm agglutination, precipitation of hyaluronidase). The inhibiting action is highly specific, thus antirabbit semen antibody failed to inhibit cumulus dispersing action (CDA) and hyaluronidase activity of bovine testicular hyaluronidase and CDA of guinea pig, mouse or rat sperm. Human semen hyaluronidase disperses rabbit cumulus and depolymerizes hyaluronidase. Both of these effects are inhibited by rabbit antihuman semen antibodies.

To test for isoantibody formation five virgin female rabbits were injected, 2 with rabbit semen and 3 with rabbit epididymal sperm, in Freund's adjuvant emulsion (protocol of

Menge, 1968, used to produce infertility). Post injection globulins (both native and univalent) from all five females inhibited CDA and hyaluronidase activity of rabbit ejaculated sperm. Globulin from two of the rabbits were also examined and found to inhibit CDA and hyaluronidase activity of epididymal and capacitated sperm.

This is the first case of a well known mammalian sperm substance with recognized function in reproduction that is inhibited by antibodies of heterologous and isologous origin. The isoantigenicity of the hyaluronidase and inhibitory activity of the isoantibodies suggests that hyaluronidase may be involved in some cases of immunologically induced female infertility and is a potential infertility agent.

Research aided by NICHD contract No. 70–2253 and Population Council grant No. M 70-144C.

Trichoplax adhaerens *Schulze, 1883: return of an enigma.* Richard L. Miller.

Trichoplax, as described by F. E. Schulze (*Zool. Anz.,* **6**), is a multicellular, gray-white, highly flattened organism with an irregularly shaped, highly mobile periphery, possessing three cell layers: (1) a dorsal squamous layer bearing scattered cilia and unusual, characteristic "shiny balls" (Glanzugeln) of 5–8 micra in diameter; (2) a ventral columnar layer, uniformly ciliated and bearing smaller refractile balls; (3) a middle "mesenchyme" layer composed of amoeboid cells bearing "lumpy" inclusions. The middle layer cells are set in a narrow fluid-filled space between the dorsal and ventral layers. The animal moves by gliding over the substrate, mainly through the action of the ventral clilia, changing shape constantly by contracting and extending portions of the edge of the body. Narrow folds frequently appear in the margin as the animal lifts a portion of its body off the substrate. The only axis of polarity is dorso-ventral and the animal is asymmetrical about this axis. No organs are present, the animal lacks muscle bands and the mode of feeding is unknown. Reproduction is asexual by fission and fragmentation. Further observations of a similar nature were published by Schulze (1891, *Abh. Akad. Berlin*), Garbowski (1903, *Morphogenetische Studien*), Monticelli (1893, *Mitt. Zool. Sta. Neapel,* **12**), and Stiasny (1903, *Z. Wiss. Zool.,* **75**), the latter author describing swimming behavior in small *Trichoplax.*

In 1908, Krumbach (*Zool. Anz.,* **31**) claimed that *Trichoplax* is a modified planula larva of the hydromedusa *Eleutheria krohni* Krumbach. Although this report was strongly disputed by Schulze (1914, *Zool. Anz.,* **44**) and effectively refuted by Schubotz (1912, *Zool. Anz.,* **39**) Hyman (*The Invertebrates, Vol. 1,* page 243) supports the claim of Krumbach. This is apparently the last mention of *Trichoplax* in the literature.

An unusual organism, found on the walls of marine aquaria at Temple University and now under culture, agrees in every detail with the published descriptions of *Trichoplax.* The available histological and behavioral evidence supports the conclusions that the animal is not a coelenterate and lacks all flatworm characteristics except the dorso-ventral flattening. That such an organism can survive in the free-living state may have profound implications for current views of the origin and phylogeny of the lower metazoa. Its ultimate classification must await determination of the complete life cycle.

Motility control mechanisms in Arbacia *sperm.* Leonard Nelson.

Spermatozoa function as multiple subunit single cell excitable systems, exhibiting spontaneously fluctuating periodic membrane potentials and a high acetylcholinesterase (AChEase) activity. To determine whether a causal interaction exists between this enzyme system and flagellar wave production, *Arbacia* sperm were systematically exposed to a variety of agents which influence acetylcholine (ACh) metabolism or cell membrane stability. In each case a dose-dependent response and occasionally biphasic effect on rate of sperm movement resulted. Eserine and DFP caused 50% slowing at 1 mM and about 50% acceleration at 1 μM. But ACh itself over the same concentration range gave only ± 10% variance from the untreated control swimming speed. This may be attributed to the difficulties encountered by such highly polar quaternary NH_4^+ molecules in entering the cell. While 20 mM DMSO per se had no effect on motility, its presence apparently facilitates ACh penetration and initially nearly doubles the sperm speed; then destruction by the intracellular AChEase soon restores swimming to the control rate. This observation supports the hypothesis that a critical level of ACh and its rapid hydrolysis are involved in sperm motility regulation. Hemicholinium depresses sperm motility at all concentrations above 100 μM; DMSO only slightly potentiates this action, suggesting that the sites of ACh synthesis or storage must be located close to the cell surface. Procaine, which stabilizes cell membranes, initially excites the cells to more than double the control swim speed at an optimum of 1 mM while causing 50% inhibition at 10 mM. Longer exposure causes complete cessation of motility at these concentrations. On the other hand strychnine, which lowers the excitability threshold by tending to depolarize the cell membrane, has prolonged effects over a broader range (50% slowing at 5 mM and 50% speeding up at 10 μM). We conclude that sperm cell activity may be modified if externally neurotropic agents can penetrate the cell to act on intracellular membrane depolarizing, regulatory systems.

Supported under research contract NIH 70-2313 and USPHS research grant 5-RO1-HD-03266.

Microsporidia in the reproductive tract of Libinia dubia. Muriel H. Walker and Gertrude W. Hinsch.

A microsporidian parasite, *Nosema* sp., has been found in the epithelium of the vas deferens of the spider crab *Libinia dubia*. Cells were heavily infected with mature and developing spores. The earliest stage observed is the spore mother cell. This cell possesses a dikaryon. The nuclear membrane is double and the space between the two layers is uneven. Along the length of the nuclear membrane are electron dense areas which may represent a simple form of nuclear pore. Within the nucleus microtubules of the intercellular mitotic spindle are observed. Occasionally, clumped nuclear material is seen which may represent the chromosomes linking up on the spindle. The sporont undergoes cytokinesis to form the early sporoblast. These cells possess a dikaryon. A centriolar plaque may be observed at the nuclear surface. The first structure of the mature spore to form is the polar filament. This develops in association with a modified Golgi apparatus consisting of numerous small cisternae frequently filled with electron dense material. Also associated with the Golgi are large spherical masses of similar material, sometimes surrounded by several layers of membrane. The polar filament is formed as two hollow tubes, the inner tube being excentrically placed within the outer one. Preparations treated with the silver nitrate methenamine technique for the detection of complex polysaccharides show a positive reaction in the Golgi, the spherical masses and associated membranes and in the polar filament. The developing spore wall also shows a positive reaction. Infected cells show degradation of the cytoplasmic organelles and large numbers of coated vesicles are found.

Work supported by a grant (F-70-UM-6A) from the Florida Cancer Society. Dr. Hinsch is an NIH Career Development Awardee.

Effects of near UV tryptophan photoproducts on proteins. Seymour Zigman.

When tryptophan is irradiated with near UV light, colored products are formed within a few hours (exposure at 3000 μw/cm^2). Differences from tryptophan of one purified product obtained by passage of near UV tryptophan through sephadex G10 columns were found. The product appeared to be twice the molecular weight of tryptophan; absorbed far UV light at a lowered wavelength maximum; emitted a new fluorescence at 410 nm (excited at 360 nm); no longer had free alpha amino groups; and moved as a single spot more slowly than tryptophan on paper chromatography. This product has a tentative elemental analysis of $C_{17}H_{18}N_3O_8$, and probably is a three-ringed structure of two outer phenolic rings connected through an O and a N. Several carbonyl oxygens are suspected.

This product binds easily to and colors many proteins, such as albumin, collagen, and eye lens proteins. Since it combines also with polylysine and polyarginine to give colored polymers, it must attach to NH_2 groups. A schiff base type linkage may be indicated. Properties of a purified calf lens gamma crystallin (by isoelectric focusing) were altered by combination with near UV tryptophan products. Loss of a sharp 278 nm absorption maximum and a new 440 nm fluorescence (360 excitation) were found. As a result of this reaction, the altered gamma crystallin becomes more soluble in aqueous ethanol and more electronegative (its isoelectric point drops to below 6.0).

Newer information indicates that the aqueous humor and whole lens *in vitro* became pigmented yellow-brown similarly with or without additional tryptophan when exposed for longer periods of time. The plasma proteins in the calf aqueous humor so treated also became pigmented as above, and also became more electronegative than the same proteins obtained from untreated aqueous humors.

The data suggest that the increasing coloration of human lenses with aging could be the result of the entry of near ultraviolet light of sunlight into the eye leading to changes as stated above. Experiments to prove that such reactions can occur *in vivo* are in progress. If so, aging cataracts may be partly derived from eye exposure to the near UV components of sunlight.

Near UV tryptophan also combines with RNA and DNA, and inhibits protein synthesis of lenses and retinas *in vitro*, and may be some type of an antibiotic.

Supported by Rochester Eye and Human Parts Bank, and the PHS (grant # EY-00459).

GENERAL SCIENTIFIC MEETINGS

AUGUST 23–26, 1971

Effects of multiple antibody layers on the morphology and fertilizability of sea urchin eggs. NEIL R. ACKERMAN AND CHARLES B. METZ.

Rabbit antibodies against sea urchin eggs (R × SU) wrinkle the egg surface and inhibit fertilization. Univalent (papain digested) antibodies do not (Metz and Thompson, 1967). Effects of multiple antibody layers on egg structure and fertilization are reported here.

One-tenth ml of dejellied eggs was incubated one hour in 0.3 ml of 1% bivalent (native) or univalent R × SU, washed and incubated in 0.3 ml of 1% bivalent or univalent sheep antirabbit globulin (S × R) for one hour. Following additional washes the eggs were treated with 0.6 ml of 1% rabbit antisheep globulin (R × S) and examined two hours later. Fertilizability was determined through a threefold sperm dilution range beginning at 9 times concentration for 100% fertilization of controls.

Bivalent antibody wrinkled the egg surface and inhibited fertilization. The response was strongest with antiegg antibody (R × SU) but also occurred when the bivalent layer was displaced from the surface by one or even two complementary "layers" of univalent antibody, *e.g.*, the sequence: univalent R × SU + bivalent S × R; or univalent R × SU + univalent S × R + bivalent R × S. The percentage fertilization following bivalent antibody treatment never exceeded 10, even when displaced by two "layers" of univalent antibody; over 85% of control or univalent antibody (one or even two "layers") treated eggs routinely fertilized. Iodine^{125} and fluorescein labeling confirmed the association of R × S globulin with egg.

These data indicate that (1) univalent antibody molecules bind to eggs; (2) even two univalent antibody "layers" (*e.g.*, univalent R × SU + univalent S × R) do not affect egg morphology or fertilizability and (3) cross linking of egg antigens by bivalent antibody directly (R × SU), secondarily (univalent R × SU + bivalent S × R) or tertiarily (univalent R × SU + univalent S × R + bivalent R × S) produced wrinkling and fertilization inhibition.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant 5-TO1-HD00026–09).

Mechanistic biology and physics, 1900–1930. GARLAND E. ALLEN.

Throughout its history biology has derived considerable impetus from both the content and methodology of the physical sciences. In the late nineteenth and early twentieth centuries this took two forms. The first was the strongly mechanistic-reductivist movement associated with the work of Jacques Loeb; the second was the holistic, "organic mechanism" developed by L. J. Henderson. Loeb's work on artificial parthenogenesis and tropistic movements in insects convinced him that all life phenomena could be reduced to physico-chemical interactions. Other workers at the time, such as T. H. Morgan or W. J. V. Osterhout agreed with Loeb's biological views, and tried to encourage all biologists to use the mechanistic approach. What Loeb and others had in mind when they talked about using the methods of physics and chemistry was essentially the pre-quantum mechanics notion of atoms as hard, impenetrable objects interacting on a purely mechanical level. Like most pre-quantum physicists, biologists who adopted the "physical" or mechanistic mode of thought were largely philosophical materialists. They believed that the physical universe had an existence independent of the observer; the function of natural science was to discover the nature of this reality. Atoms and genes were not conceptual inventions, but real, physical entities in the universe.

L. J. Henderson took another approach in the 1930's. He studied the blood buffer system in terms of physical chemistry, but concluded that a mere description of the 7 or 8 separate factors involved in maintaining blood pH was insufficient to show how the system as a whole functioned. What was important was the organization of the system—the interrelationships between parts. This could only be described symbolically, by mathematical or graphical means. Thus, the relationships themselves could not be considered material entities, a realization which led Henderson to adopt by the 1930's a phenomenological philosophy. According to this view, it is impossible to ever know what reality is; all we can know is our sense impressions. Hence it is philosophically erroneous to claim too much for the entities (atoms, genes) we may need to invent to bring order into our experience. This view found support in the physical sciences of the early twentieth century, especially that associated with the rise of quantum theory. Ernst Mach's philosophy (developed before the quantum theory) led the way, but was echoed in the writings of Bohr and Schrödinger. Quantum theory threw into doubt the classical structure of the atom, and through Heisenberg overthrew the traditional views of cause and effect. To Mach and others, changing views on the nature of atoms showed that there was no reality independent of the observer. Henderson, through his friend Alfred North Whitehead (both were at Harvard), was greatly influenced by this view, as were also William Bateson, C. S. Sherrington, W. B. Cannon, and W. M. Wheeler. While they all opposed vitalism, they rejected the simplistic mechanism of Loeb, and tried to steer biology away from a purely reductivist, materialistic course.

Sodium and potassium influxes in Tetrahymena. R. S. Beauchamp, S. A. Hilden, D. L. Kropp and P. B. Dunham.

Tetrahymena generally maintains a concentration of K higher than that of the environment. The intracellular concentration of Na, $(\mathrm{Na})_o$, is maintained constant at 5 mM/kg cells for $(\mathrm{Na})_o$ ranging from 3 to 20 mM/l. These facts suggest that *Tetrahymena* possesses the following system for regulating cations: active K influx and both active efflux and active influx of Na. Active K influx and active Na efflux had been shown previously in *Tetrahymena*. We have examined Na influx and the relation between Na influx and K influx.

Tetrahymena pyriformis in late log phase was collected by gentle centrifugation, washed and incubated for 2–3 hours in medium consisting of $\mathrm{CaCl_2}$, $\mathrm{MgSO_4}$, and histidine, all at 0.5 mM/l. To this medium was added various concentrations of Na and K, depending upon the particular experiment. Unidirectional influxes of Na and K were measured using the appropriate isotopic tracers.

The unidirectional influxes of both Na and K as a function of their respective external concentrations show saturation kinetics, suggesting carrier mediated influx. In both cases there was also a component of influx which was linearly related to the external concentration and which probably represents passive diffusion. Further evidence for active Na influx are the net Na influxes against the electrochemical potential gradient demonstrated at low $(\mathrm{Na})_o$.

Na and K are mutually inhibitory of their respective influxes, apparently by specific interaction with transport sites. Two additional kinds of evidence are consistent with, but not proof for, separate pathways for active Na and K influx: (1) the V_{max} for Na influx is one third that for K influx ($\mathrm{Na} = 0.22$ mM/min × kg cells; $\mathrm{K} = 0.66$). (2) Rb, a competitive inhibitor of K influx, inhibits Na influx to a smaller extent than K influx. At 0.2 mM/l external Na or K and 10 mM/l Rb, K influx in inhibited by 75% while Na is inhibited by 50%.

This research was supported by U.S.P.H.S. grant NS-08089.

Properties of junctions mediating electrical coupling between embryonic cells. M. V. L. Bennett and M. E. Spira.

Cells from blastulae of killifish *Fundulus* were isolated and assembled in pairs. The cells adhere and generally become electrically coupled, often very closely. Colchine blocks mitoses but has no obvious effect on adherence or coupling. Fluorescein injected iontophoretically into one cell of a pair does not spread to the other cell. In contrast fluorescein and even larger molecules spread between coupled cells in a number of adult tissues. Theoretically the cell pairs could be coupled by way of specialized junctions between them or by way of extracellular space if the appositional region were surrounded by a *zonula occludens* (truly tight junction) and the apposed membranes were of somewhat lower resistance. Two lines of evidence support coupling by way of specialized junctions. First, fluorescein can enter the cells from the outside, and since it does not spread between cells when injected in one, the extracellular space between them ought to be open to the outside in order to provide an effective shunt to spread of dye. Secondly, glutaraldehyde fixation greatly increases coupling resistance, *i.e.* decreases coupling. Similar results are obtained with electrotonic junctions that couple cells of the crayfish septate axon. Suitable controls indicate tonicity changes and buffers are not responsible for increased junctional resistance. In contrast fixation of the intact blastula or gastula shows little if any effect on leakage through extracellular space between cells of the enveloping layer, an epithelium of cells joined by *zonulae occludentes*. These results indicate that junctions coupling embryonic cells can be less permeable to larger molecules than junctions in adult tissues. The lower permeability may facilitate independence of growth and differentiation while allowing metabolic sharing and transmission of nutrients. It is also possible that the small ions to which the junctions are permeable carry information important to development.

Supported partially by N.I.H. Grant HD02428.

Cellular differentiation in the planula of Aurelia. Beverly H. Bergstrom and Gertrude W. Hinsch.

Spermatozoa of male *Aurelia* are shed into the sea water and fertilization occurs in the female. Zygotes develop to a planula stage in modified brood pouches in the oral arms. Segments of the oral arms and planulae from the surrounding sea water were fixed in paraformaldehyde—glutaraldehyde and osmium and embedded in Spurr for electron microscopy. The planulae averaged $300\,\mu$ long and $134\,\mu$ wide. A ciliated ectoderm and a distinct endoderm were evident. The ectoderm to become the basal region was highly vacuolated.

Ultrastructurally the planulae possess a surface epithelium of tall columnar cells with numerous cilia and microvillae. In the basal areas are various gland cells containing large granules of various densities, which are probably secretory in nature, were seen. Long rows of vesicles suggestive of primitive nerve synapses were sandwiched between adjacent cell membranes. The cnidoblasts with developing nematocysts were scattered beneath the epithelial surface. Their capsule walls were very thick with scalloped outer membranes. In planulae studied no nerve cells or myofibrils were seen.

In some planulae a cavity was apparent in the endodermal region. The endodermal cells contained numerous large vacuoles. Various granules were scattered through the layer. Most yolk had been absorbed.

The mesoglea between the endoderm and ectoderm layers was non-cellular. It contained a fibrous material which branched between cells of both layers. It was also fairly homogeneous in density.

This study was supported by NIH grant 5-T01-HD00026-10 to the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory.

Mechanical properties of the sheath and axoplasm of the squid giant axon. S. F. Borg.

Two separate experiments and the associated theoretical equations permit the determination, for the first time, of particular elastic-mechanical properties for the squid axon sheath (*i.e.*, membrane, Schwann cell and connective tissue complex) and also for the axoplasm.

These are, (1) a tension deformation test, (2) an internal pressure-deformation test. The first experiments were performed at M.B.L. Data for the second experiments were extracted from a paper by Vargas dealing with filtration coefficients of axons.

Isotropy may reasonably be assumed. Notation is—S is sheath, A is axoplasm, E is modulus of elasticity, ν is Poisson ratio and h is the thickness of the sheath. From (2), $hE_S = 29600$ dyne/cm and $\nu_S \cong \frac{1}{2}$ are determined. Using these, from (1), assuming the sheath and axoplasm deform as a unit, $E_A = 0.5 \times 10^7$ dyne/cm^2 and $\nu_A \cong \frac{1}{2}$ are obtained. Note that a separate value for E_S could not be obtained although hE_S could be determined. Thus a sort of indeterminancy condition prevails. The values $\nu \cong \frac{1}{2}$ correspond to incompressible action for the sheath and axoplasm.

If one assumes $h = 20\ \mu$ (a value obtained by Cole) then $E_S = 1.5 \times 10^7$ dyne/cm^2.

The values for E_S and E_A are of the same order of magnitude as those obtained by Katchalsky and also Rand for the red cell membrane.

One may speculate on the role played by the velocity of infinitesimal pressure-tension pulses in the behavior of axons. The velocity of longitudinal pulses is $v = (E/\rho)^{\frac{1}{2}}$, ρ being the density of the "bar." In the present case one obtains $v_A = 20$ m/sec and also the ratio $v_S/v_A \cong 2$. These values suggest correlations with the velocity of the action potential and also the on-off time constants of the Na and K ions.

Financial support was received from NSF Advanced Training Program in Membrane Biophysics, Grant #GZ1918. The assistance of Drs. W. J. Adelman and K. S. Cole is also acknowledged.

Density gradient centrifugation as an aid in sorting planktonic organisms. RICHARD A. BOWEN, JEANNE M. ST. ONGE, C. A. PRICE, AND JOHN B. COLTON, JR.

We find that ichthyoplankton (fish eggs and fish larvae) can be separated from other invertebrate zooplankton by isopycnic centrifugation in gradients of sucrose and silica; sorting of other classes of zooplankton may also be possible.

Preserved samples of invertebrate zooplankton, fish eggs, and fish larvae, representing a typical assortment of marine plankton, were layered over linear gradients of 0–60% w/w sucrose or 0–15% w/w silica ("Ludox AM," kindly supplied by E. I. DuPont & Nemours) in 100-cc swinging buckets and centrifuged for one hour at 1000 rpm in a size 2 (IEC) centrifuge. In sucrose gradients the invertebrate zooplankton were confined to the two ends of the gradient, while 85% of the fish eggs were recovered from an intermediate zone (27.5% to 55% w/w) with an enrichment of about 13. In "Ludox AM" the fish eggs banded in a narrow region between 2% and 3% (w/w) while fish larvae banded at the bottom of the gradient between 10% and 14% w/w. Of the six dominant classes of plankters, only *Salpa* overlapped appreciably with the fish eggs and none overlapped with the fish larvae. Thus, of the gradients tested, "Ludox AM" offers the most advantages; sucrose may also be useful for subfractionation. Gradients of sodium bromide and dextran were found to be totally unsuitable.

The research was supported by a grant from the U. S. Department of Commerce, National Oceanic and Atmospheric Administration.

Na^+-dependence of reversal potential of light-induced current in Limulus *ventral photoreceptors.* JOEL E. BROWN AND MICHAEL I. MOTE.

When NaCl was replaced partially by sucrose in the artificial sea water bathing a ventral photoreceptor, reversal voltage (V_R) of the light-induced current became more negative. Stimuli were 100 msec long, 1 per 30 sec. $[Na^+]_{out}$ was varied from 107 to 670 mM. Curves of V_R versus log $[Na^+]_{out}$ had steepest slope near $[Na^+]_{out} = 430$ mM; slopes were 41–64 (mean $= 55$) mv/decade decrease in $[Na^+]_{out}$. The curves could be approximated by straight lines having slopes of 41–52 (mean $= 50$) mv/decade. With replacement of Na^+ by $TRIS^+$, the slopes were 44–58 mv/decade; with replacement of Na^+ by choline$^+$, the slopes were 18–31 mv/decade; and with replacement of Na^+ by Li^+, the slopes were 0 mv/decade. For holding voltage at resting potential, light-induced currents evoked by flashes of mixed intensity were nearly proportional to $[Na^+]_{out}$, for partial replacement of Na^+ by Li^+, choline$^+$ and $TRIS^+$; the plots intersected the zero Na^+ axis at inward values of current. With replacement of NaCl by sucrose, for holding voltage at resting potential the currents evoked by fixed intensity flashes also were proportional $[\mathrm{Na}^+]_{\mathrm{out}}$; however, the plots intersected the zero Na^+ axis at outward values of current. The data are consistent with the hypothesis that the light response is generated principally by a light-induced change in membrane conductance to the sodium ions; Li^+ (and less well, choline$^+$) can substitute for Na^+ as the charge carrier during the light response.

Supported in part by NIH grants EY-00151, EY-00312 and EY-00377.

Sponge aggregation I: Are carbohydrates involved? Max M. Burger, Stanley M. Lemon and Ronald Radius.

Some sponge cells were shown to aggregate species specifically. Humphreys and Moscona have isolated a 100 S surface particle from sponges which contains carbohydrate and proteins and seems to be required for species specific reaggregation of dissociated sponge cells.

The involvement of carbohydrate in the best studied aggregation system (*Microciona prolifera*) is indicated from the following findings.

1. Half maximal inhibition of aggregation (at 18° C, without factor) could be shown with 5×10^{-3} M buffered glucuronic acid. The same concentration inhibited also aggregation of chemically dissociated cells at 4° C with factor. Galacturonic acid, sialic acid and the other monosaccharides occurring on animal cell surfaces had no inhibitory effect. The mucopolysaccharides heparin and hyaluronic acid had no pronounced effect either. Aggregation of *Cliona celata* was not inhibited with glucuronic acid, indicating that the inhibition was species specific.

2. *Helix pomatia* extracts containing glucuronidase inactivated both the aggregation of *Microciona* cells at 18° C without, and at 4° C with factor. Since glucuronic acid (0.1 M) was the only monosaccharide that inhibited the effect by this enzyme mixture competitively one can conclude that the *Helix pomatia* effect was due to its glucuronidase content.

3. Mucin was the only glycoprotein so far found to inhibit *Microciona* aggregation. After periodate treatment (5×10^{-4} M) partially repurified mucin lost its activity but not after sialidase treatment. Partially purified oligosaccharide fragments of mucin obtained by exhaustive pronase treatment were powerful inhibitors. In a mixed reaggregation or sorting out experiment in the presence of mucin only *Cliona* cells were forming aggregates but not *Microciona* cells.

4. Exhaustive treatment of *Microciona* factor with proteases led to a carbohydrate fraction which inhibited the aggregation of *Microciona* cells very strongly but not that of *Cliona* cells.

Aided by USPHS grant CA-10151.

Preliminary study by scanning electron microscopy of dissolution of the shell of Mytilus *by the accessory boring organ of* Urosalpinx. Melbourne R. Carriker.

Excavation of boreholes in the shell of bivalve prey by *Urosalpinx cinerea* (Muricidae, Gastropoda) is accomplished by a complex process in which the accessory boring organ (ABO) secretes a little-known substance which dissolves shell, and weakened shell and debris are removed by the radula and swallowed. Penetration is achieved by successive alternation of long periods of chemical activity and short periods of rasping. Chemical activity dissolves the bulk of the borehole shell and shapes the borehole, whereas radular activity plays only a minor role in shell removal.

This paper reports the preliminary results of a study of the action of the ABO secretion of *U. c. follyensis* on the ultrastructure of the shell of *Mytilus edulis* viewed in fracture sections of incomplete boreholes. Boreholes were removed from snails before they were completed, dried, cracked open over a fulcrum, shadowed with palladium-platinum in vacuum and examined with a Joelco scanning electron microscope at magnifications ranging from 50 to 14,000 ×.

The periostracum, prisms of the outer prismatic stratum, and prisms of the inner nacreous stratum are distinctly different. Both the organic and calcareous components of shell are readily dissolved by the ABO secretion. Solution of the prismatic regions proceeds progressively through the organic prismatic envelopes into the prisms, leaving partly exposed tapering prisms in the borehole wall. Dissolution of the nacreous region advances in a less ordered pattern.

In both types of prisms dissolution exposes nodular structures of various sizes. These are more conspicuous in the nacreous region, and grade in form from complete to partial nodules from the exterior of the borehole wall into the prisms. A coat, probably a combination of ABO secretion and dissolved shell, forms over the borehole surface during chemical activity. Nodules, similar in size and shape to those in the partially dissolved prisms, occur on the coat. The coat may provide a means for at least partial removal of the products of dissolution by the radula.

Mr. Dirk Van Zandt assisted in the preparation of the fracture sections, and Dr. Virginia Peters collaborated in the use of the scanning electron microscope. Supported in part by Public Health Service Research Grant DE 01870 from the National Institute of Dental Research; SEP Contribution No. 251.

Pharmacology of horizontal cells in the isolated perfused skate retina. LUIGI CERVETTO AND EDWARD F. MACNICHOL, JR.

Short chain amino acids have been shown to modify the excitability of nerve cells. Indeed some appear to act as synaptic transmitters.

Aspartate has been shown to abolish all of the ERG except the receptor component.

In the present study Na-L-Aspartate, Na-L-Glutamate and GABA were added to a buffered Ringer solution flowing over both sides of the retina while recording the intracellular response to light from horizontal cells and the ERG. We could hold the intracellular recording long enough to obtain a constant effect of the test solution and reverse this effect by washing out the preparation with the control bathing medium.

When the retina was exposed to a test solution containing one of the three aminoacids at a concentration of 20 mM the horizontal cell membrane was depolarized, until finally the hyperpolarizing response to light was completely suppressed.

The effects of Aspartate differed somewhat from those of Glutamate and GABA. While the concentration of Aspartate was increasing, both the amplitude of hyperpolarizing response to light and its time constant were increased. The cell membrane becomes increasingly depolarized, the depolarization finally reaching a stable level at which the response to light disappeared, or often inverted its polarity. In contrast the actions of Glutamate and GABA were progressively to depolarize the cell membrane and to decrease the hyperpolarizing response produced by light.

Our preliminary experiments are consistent with the hypothesis that a transmitter released in darkness keeps the cell hyperpolarized by increasing sodium conductance, and that the effect of Aspartate is to decrease the conductance to potassium and/or chlorine.

Both authors are from the National Institute of Neurological Diseases and Stroke, Bethesda, Maryland.

Analysis of photosynthetic membranes by acrylamide gel electrophoresis. RODERICK K. CLAYTON AND ROBERT HASELKORN.

The pigmented membranes of photosynthetic bacteria have been analyzed by electrophoresis in polyacrylamide, following their dissolution by exposure to sodium dodecyl sulfate (SDS) and mercaptoethanol. With *Rhodopseudomonas* (*Rps.*) *spheroides* the protein of the component already identified as the photosynthetic reaction center yielded three subunits of apparent molecular weights 19,000, 23,000, and 27,000. The density of staining suggested a ratio of 1:1:1 for these subunits. The subunits of reaction center protein could not be found in any preparation made from the pigmented but non-photosynthetic mutant strain PM-8, of *Rps. spheroides.* Triads of bands suggesting reaction center protein subunits could be discerned in preparations from *Rhodospirillum rubrum, Rps. capsulata,* and *Rps. palustris,* but not *Rps. gelatinosa* or *Rps. viridis.*

The coomassie blue (CB) used as a stain was observed to be fluorescent on some bands in the gel and not on others. With the "reaction center triad" the heaviest band was fluorescent and the others were not; this set of bands was accordingly very easy to recognize in crude preparations. We entertain the hypothesis that the fluorescence of CB can be quenched by SDS retained in the gel by some of the protein subunits; perhaps by the more hydrophobic ones.

Ascidian metamorphosis: Contractile tissues, cytochalasin B and hydrostatic pressure. RICHARD A. CLONEY, JAY LASH AND RONALD R. MINOR.

During tail resorption (TR) in the aplousobranch ascidians *Amaroucium constellatum, Distaplia occidentalis* and *Diplosoma macdonaldi* the caudal epidermis becomes contractile and forces the underlying tail tissues into the trunk. In all three species arrays of 50–70 Å microfilaments become rapidly aligned in the outer portion of each caudal epidermal cell. In the stolidobranch *Botryllus schlosseri* aligned filaments are found in the basal portion of the epidermal cells. In the stolidobranch *Boltenia villosa* aligned filaments are found in the notochordal cells. These arrays are thought to be contractile organelles. Cytochalasin B (CCB) blocks the initiation of TR and reversibly inhibits its progress once begun. The longer the treatment the longer it takes the tissues with filaments to regain contractile properties. In *A. constellatum* contraction of the epidermis could be inhibited and allowed to recover up to four times with successive application and removal of the drug. CCB disrupts the organization of the arrays of microfilaments when it blocks contraction. The organization of the filaments returns when the drug is washed away. The effective concentration differs in the three suborders (aplousobranchs, 3 spp., 0.25–1 μg/ml; phlebobranchs, 2 spp., 1–2 μg/ml; stolidobranchs, 5 spp., 5–20 μg/ml). Hydrostatic pressure of 6500 psi also reversibly blocks TR in *D. occidentalis,* and the arrays of filaments become disorganized in these specimens. CCB reversibly blocks retraction of adhesive papillae and the pulsation of ampullae. Arrays of filaments in these organs are found near the basal surface of the epidermal cells.

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Inter-receptor relations in the retinas and optic lobes of the squid, Loligo pealii*: an electron microscopic investigation.* ADOLPH I. COHEN.

Animals were fixed by perfusion. Confirming earlier investigations, photoreceptor outer segments exhibit a square or rectangular cross section, with alternating smooth and microvillous borders. Villous borders may oppose villous or non-villous borders of adjoining cells, appositions of smooth borders being rare. Microtubules were found close to the cell membrane and predominantly on the villous sides. Cross-sectional area and shape were stable in a given cell over approximately 100 microns of outer segment height and in a population at a given level, areas exhibited a skewed, unimodal distribution with small cross sections of 0.06 microns^2 being most frequent. Increases in cross-sectional area were generally associated with an increase in the length of villous sides. With certain fixations, apparent fusions of microvillous or inner segment membrane of adjoining photoreceptors were seen, but these are likely to be artifactual.

Confirming earlier investigators, there are extensive groups of paired, cytoplasmic membranes in the somal and subsomal regions of the photoreceptors. Cross sections at this level suggest the ordering of groups of 8–10 cells based upon each member's cytoplasmic membranes contributing one radius to a spoke-like arrangement. The membranes are largely screened by melanin at outer retinal levels in glia and photoreceptors.

The sub-retinal plexus exhibits pre-synaptic and synaptic profiles with clear, dense core, or mixed vesicles. Efferent versus postulated inter-receptor endings were not distinguished with certainty.

Ensheathed photoreceptor axons enter the optic lobe and penetrate the outer nuclear layer to end in large, carrot-like terminals filled with clear vesicles. The uppermost terminal regions were often deeply invaginated by large processes from adjoining terminals and short chains of joined cells could be seen. These junctions did not exhibit the conventional morphology of chemical or electrical synapses. The remaining terminal exhibited numerous invaginated but conventional small, post-synaptic endings. Rare superficial pre-synaptic endings on terminals were seen, but no tunnel fibers as reported for octopus.

This investigation was supported by grant EY 00258 of the National Institutes of Health, and the investigator by Career Development Award NB-3170.

Changes in axon fluorescence. L. B. COHEN, H. V. DAVILA AND A. S. WAGGONER.

In an effort to understand the changes in axon structure which underlie action potential propagation, we have measured changes in fluorescence of dyes bound to giant axons from the squid *Loligo pealii.* Each fluorescence change depended in some manner on membrane potential and not on ionic current or membrane conductance. With most dyes, including ANS, Acridine Orange, Brilliant Cresyl Blue, Neutral Red, Neutral Violet, Phosphine GN, Pyronin B, Quinaldine Red, Quinoline Blue, Rhodamine B and TNS, the fluorescence changes were linearly related to membrane potential. However, Coriphosphine O and tetracycline gave fluorescence changes that were dependent on the square of potential, much like the potential dependent light scattering and birefringence changes.

The fluorescence changes measured with ANS microinjected into the inside of the giant axon were opposite in sign to those found with ANS added to the outside of the axon. ANS was unique in this respect, for the fluorescence changes found with Acridine Orange, chlorotetracycline, Neutral Red, Neutral Violet, Pyronin B, Rhodamine B and tetracycline were independent of the side of the membrane to which the dye was added.

Several structural variations of the dye Neutral Red were studied. When the NH_2 group was converted to a two carbon amide with acetic anhydride, the fluorescence change was abolished. But, the fluorescence change was restored in the four and eight carbon amide, suggesting that the additional hydrophobicity could counteract the effect of the amide group. When molecules with different central atoms were compared, it was found that replacement of one nitrogen by a carbon atom (Coriphosphine O) or sulfur atom (Toluidine Blue O) caused a reduction in the fluorescence signal.

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A transient outward membrane current in repetitive-firing axons. JOHN A. CONNOR.

Axons which fire repeatedly in response to a maintained, suprathreshold current stimulus, termed repetitive axons, and axons which fire only once to this form of stimulus, termed non-repetitive axons, have been studied by means of the sucrose gap technique. Repetitive and non-repetitive axons with diameters of 40 to 70 microns were dissected from the walking legs of the blue crab, *Callinectes sapidus*, and lobster, *Homarus americanus*. Larger non-repetitive axons, diameter 100 to 130 microns, were isolated from the lobster esophageal commissure. The central or active node of the sucrose gap was made to be approximately 50 microns for studying the walking leg axons and up to 90 microns for the lobster giants. For voltage clamp studies the axons were treated with tetrodotoxin (10^{-6} M) in order to eliminate the large inward current transient. The general characteristics of outward membrane current under voltage clamp are identical for non-repetitive walking leg fibers and the lobster giant fiber; that is, the current develops along a sigmoid path to a steady level. Appreciable activation does not occur until the membrane voltage is made more positive than the action potential threshold. In repetitive axons a transient outward current first becomes apparent when membrane voltage is stepped to values 10 to 15 mv on the negative side of the spike threshold. The amplitude of the transient increases as the membrane voltage is stepped to more positive values. It ranges from 0.1 to 0.2 mA/cm^2 in the neighborhood of the spike threshold. For large positive-going steps the outward current record may display two maxima, the second being due to the development of delayed outward current. The outward transient reaches a peak 3 to 5 msec after the membrane voltage step is made. Decay, at voltage levels around threshold, occurs in 30 to 50 msec. The amplitude of the transient diminishes as the holding potential is made more positive and extinction occurs when the holding potential is within a few mv of the spike threshold. This transient can account for many of the subthreshold phenomena observed in repetitive-firing axons.

This research was supported by a Grass Foundation Fellowship.

In vitro *maturation of* Arbacia punctulata *oocytes.* CAROLYN M. CONWAY AND CHARLES B. METZ.

Many germinal vesicle oocytes capable of maturation *in vitro* are present in *A. punctulata* ovaries during the 24 hour period following induced shedding of mature gametes. Excised ovaries from shed urchins are placed in Tris-buffered (pH 8.0) artificial sea water containing antibiotics and cut into small pieces. The suspension is agitated for several minutes releasing germinal vesicle oocytes from the ovary. Using a dissecting microscope and micropipette germinal vesicle oocytes of ovulatory size are isolated into covered dishes containing Tris-buffered artificial sea water and antibiotics and incubated at 23–25° C. Oocytes of ovulatory size are characterized by a large germinal vesicle exhibiting a promient nucleolus. The onset of maturation is indicated by an excentric location of the germinal vesicle in the oocyte. The first and second polar bodies are extruded and the haploid pronucleus is formed. Although all isolated oocytes do not initiate maturation synchronously, the process once initiated requires 6–7 hours for completion as previously reported by Harvey. In 7 *in vitro* maturation experiments an average of 25% of the oocytes had completed maturation in 6 hours. After 24 hours of isolation 48–84% ova were present. The maximum per cent maturation, 85%, was reached between 24–32 hours after isolation. Ova produced by *in vitro* maturation fertilized and developed normally. With this method of *in vitro* maturation oocytes at known stages of maturation can be obtained for future ultrastructural, biochemical, and physiological studies.

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Hybridization analysis of DNA replication in nurse and follicle cells in Cecropia moth. MARCO CRIPPA AND WILLIAM TELFER.

Nurse tissues and follicular epithelia in the ovaries of many insects consist of greatly enlarged, endopolyploid cells which synthesize much of the protein and RNA of the oocyte. Whether the development of endoplyploidy entails total genome replication was investigated by analyzing the reannealing kinetics of labeled DNA from the two types of cells.

Female Cecropia moths on the 14th to 18th days of adult development were injected with 500 μc of H^3-thymidine (New England Nuclear, sp.act. $\sim$ 20C/mmole) and/or P^{32} and dissected one to four days later. The nurse cells were isolated manually from the rest of the follicle in which the epithelial cells are the only sites of DNA synthesis detected by autoradiography.

The newly synthesized DNA was separately extracted from these two ovarian fractions, extensively purified and sheared by sonication. Very small amounts of this DNA ($\sim$ 1000 cpm/sample, DNA spec.act. $\sim 10^6$ cpm/μg) were permitted to reanneal at various concentrations and for different times with a large excess of sheared unlabeled DNA extracted from somatic tissues of the animal.

The reassociation kinetics of the unlabeled somatic DNA and of the newly synthesized DNA were monitored by following the optical density and the radioactivity distribution after hydroxylapatite fractionation according to the procedure of Britten *et al.* During the DNA synthetic period monitored by our labeling procedure in the nurse cells and in the follicle cells, there was no detectable replication of the highly repetitive (Cot 10^{-3}–10^{-1}) fraction of the genome which in Cecropia accounts for approximately 10–15% of the DNA. In the follicular cells the newly synthesized DNA was replicated only from the unique fraction (Cot 10^2–10^4) of the genome, whereas in the nurse cells both the unique and the intermediate fraction (Cot 10^0–10^4) were replicated.

In these ovarian cells the development of endopolyploidy entails therefore the replication of only limited fractions of the genome. In addition to this, nurse cells and follicle cells, which differ in the nature of their synthetic contribution to the egg, also differ in the fraction of the genome replicated in preparation for these activities.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant 5-TO1-HD00026-10).

Aspartate isolation of receptor potentials in the skate retina. J. E. DOWLING AND H. RIPPS.

The isolation of receptor potentials in the all-rod retina of skate by L-Na aspartate has been studied and the adaptive properties of the isolated receptor potential investigated. After immersing a piece of eyecup in aspartate Ringer (10–110 mM L-Na aspartate), the b-wave of the ERG is rapidly suppressed, but a- and c-waves appear relatively unaffected; the c-wave, derived from pigment epithelium, can then be eliminated by removing the retina from the eyecup. Intracellular recordings from horizontal cells during application of aspartate Ringer show a rapid depolarization of the S-unit with an initial increase in amplitude of the light-evoked response. Thereafter, although there is little further depolarization of the cell, light-evoked activity disappears within 1–2 minutes.

The V-log I curve of the isolated receptor response matches closely those obtained from intracellular recordings of receptor activity in other species; *i.e.,* the response amplitude reaches its maximum voltage (saturation level) at intensities only 3–4 log units above dark-adapted threshold. When exposed to steady background fields, the adaptation properties of the isolated receptor response closely resemble those of horizontal cells in skate. For example, the increment thresholds are a linear function of background luminance over at least 6 log units. Dark adaptation is rapid (5–15 minutes) after light exposures that do not bleach a significant fraction of the visual pigment, whereas it is very prolonged (~ 2 hours) following exposures that bleach substantial amounts of rhodopsin. In addition, at all backgrounds, the maximum response that can be elicited by superimposed test flashes grows with time in the light. With bright backgrounds (above saturation), there is an initial silent (unresponsive) period, but given sufficient time, light-evoked responses appear and increase in amplitude.

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Coupled transport of sodium and sugar—in vivo *evaluation in the intestine of the toadfish,* Opsanus tau. A. FARMANFARMAIAN, ALLAN ROSS, AND DENNIS MAZAL.

It has been proposed that active transport of sugars and amino acids across the brush border membrane of intestinal epithelium is dependent upon a lumen-to-cell downhill gradient of sodium. This hypothesis is directly or indirectly supported by many *in vitro* kinetic experiments (Schultz and Curran, 1970, *Physiol. Rev.,* **50**: 637–718). We have tested this hypothesis by various kinetic experiments in the upper midgut of the toadfish under *in vivo* swimming conditions.

In previous communications we have described the technique and presented evidence to show that D-glucose is actively absorbed from normal Forster-Taggart saline (150 mM Na) solutions in the lumen. Our current investigations involved parallel kinetic experiments in which the rates of absorption of glucose from normal (150 mM) and reduced (15 or 0 mM) sodium solutions were determined. These were all isoosmotic solutions in which Tris Cl, choline Cl, mannitol, or KCl were substituted for NaCl or both NaCl and $NaHCO_3$. For each saline four or five concentrations of glucose and at least 13 fish were tested. The kinetic constants K_t and V_{max} were determined from Lineweaver-Burk plots and the 95% confidence limits given in the parentheses were determined from the regression of the line according to Mather. The results for normal and Tris, choline, mannitol, and KCl substituted salines were, respectively: K_t in mM, 3.7 (3.3–4.1), 2.4 (2.0–2.7), 5.7 (4.5–6.9), 5.3 (4.1–6.5), 2.2 (1.8–3.4); V_{max} in μmoles/g wet gut/hr, 9.7 (8.4–11.7), 9.6 (8.4–11.2), 15.4 (10.4–29.4), 10.1 (7.4–15.9), 4.7 (3.6–6.6). In the case of low sodium salines the concentration of sodium at the end of the experiment remained well below that of the epithelial cell or blood, even though the lumen sodium increased.

These results do not support the proposed hypothesis since a 10-fold reduction or complete elimination of luminal sodium did not increase K_t or reduce V_{max} significantly ($P < 0.05$). The significant reduction in V_{max} in the case of KCl substitutions must be attributed to the specific inhibitory action of K which has also been observed for various *in vitro* preparations. It can be stated without equivocation that active absorption of glucose can proceed with a contraluminal Na gradient in this system.

In part supported by NSF grant GB-8089.

A comparative study of the respiratory adaptations of the podia of Echinoids. DOUGLAS H. FENNER.

Because our knowledge of Echinoid podia is limited, and because they have been shown to be the primary respiratory surface, their morphology has been examined. In examining 18 species of Echinoids, four primary respiratory adaptations were found. (1) The ciliary current entering the podium is separated from that leaving the podium for the ampulla.

(2) The surface area of the podia is increased. (3) A counter-current appears across the podial and ampullar walls. (4) Podia modified for respiration are found only on the aboral surface, where the oxygen concentration is greatest. Cidaroids and Echinothurids have 2 pores in the body wall, and hollow podia and ampullae. Camarodonts have in addition a septum in the podium which is continuous with the inner, circular layer of connective tissue, and separates the currents in the proximal half of the podium. Septa channel the currents through the flattened ampullae, which resemble gill lamellae, as do the ampullae of the following podia. Some of the podia of *Arbacia* are flattened such that a cross section is dumbbell-shaped. The ciliary current comes up the tube along one edge, passes through the thin area, and down the tube on the other edge. Strands cross the thin area. The respiratory podia of Clypeastroids and Spatangoids are shorter and longer than those of *Arbacia.* Clypeastroids' have flat surfaces, and Spatangoids have laterally folded surfaces. The lumina of both are crossed by strands in rows forming channels. In most cases, non-respiratory (locomotory, etc.) podia are also present.

I acknowledge support from NSF Grant number GZ 1902.

Some aspects of muscle development and function in Artemia salina. HENRY FINCK.

When the nauplii of brine shrimp first emerge, the muscles of their swimming appendages consist of single myofibrils that can be observed in the living state through the transparent exoskeleton. Optical conditions for phase contrast or polarization microscopy are improved, however, by soaking these faerie beasts in glycerol/sea water (50/50, v/v) at 4° C for several days, and viability is not impaired: 0.05% eosine added to the medium is excluded, while 0.05% acridine orange stains the nuclei of many cells and the myofibrils; feeble swimming movements are resumed on warming to room temperature; and complete recovery occurs within a few hours after return to sea water. During the first 2 days post-hatching the myofibrils are not cross striated and are not birefringent. Birefringence gradually increases and by the end of the first week the myofibrils are clearly cross striated, with a sarcomere spacing of about 2.5 μ. By the end of the second week of development the typical crustacean pattern has appeared and the sacromeres are 5 to 7 μ long. The entire myofibril does not shorten when the glycerinated muscle contracts: tension is maintained by a peristaltic-like passage of a contraction/relaxation wave along the length of the myofibril. Contraction appears to occur at constant volume; *i.e.,* the glycerinated myofibril thickens in the region of contracting sarcomeres. The binding of acridine orange by the functional muscles of glycerinated Artemia may provide interesting insights into the molecular interactions accompanying contraction/relaxation. When contraction waves are observed by fluorescence microscopy, relaxation/contraction is accompanied by a green/red shift in emitted light.

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A cytoplasmic interaction during the first stages of embryogenesis that plays a necessary role in the formation of light producing cells in the ctenophore Mnemiopsis. GARY FREEMAN, GEORGE T. REYNOLDS, AND J. R. MILCH.

The cytoplasm of the ctenophore egg is distributed in two phases: a central yolky phase and a peripheral clear cortical phase. If the fertilized uncleaved egg is centrifuged at 6000 *g* in a solution containing 1 part 1.1 modal sucrose and 4 parts sea water for 10 minutes the cytoplasmic phases of the egg are stratified and it frequently splits into two fragments: a centripetal fragment containing the cortical cytoplasm and a centrifugal fragment containing yolky cytoplasm. The nucleus is always found in the cortical cytoplasmic fragment.

An egg fragment which contains only cortical cytoplasm develops into an abnormal larvae which contains well developed comb plate cilia; other structures such as the apical organ, the tentacles and the stomadeum do not develop, and it does not produce light when it is stimulated. Egg fragments which contain clear cortical cytoplasm and a small amount of yolk (1–5% of the egg volume) develop into an abnormal larvae with the same external characteristics as the larvae that develop from fragments which contain only cortical cytoplasm, however these larvae produce light when they are stimulated.

The first differential division occurs at the 8 cell stage during the development of the ctenophore egg. The 4 outer cells (E macromeres) differentiate to form a cellular mass that contains the comb plate cilia cells, while the 4 inner cells (M macromeres) differentiate to form a cellular mass that contains cells that produce light when stimulated. If the blastomeres of an egg fragment containing only cortical cytoplasm are separated at the 8 cell stage frequently both the M and E macromere isolates differentiate comb plate cilia. If the blastomeres of a fragment that contains cortical cytoplasm and a small amount of yolk are separated at the 8 cell stage only the M macromere isolates produce light. Comb plate cilia are formed by the E macromere isolates and by some of the M macromere isolates that do not produce light.

These findings suggest that the yolky cytoplasm is necessary for the differentiation of photocytes and that this cytoplasm plays a necessary role in the differential division which occurs during the formation of the 8 cell stage.

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Anode-break excitation: a formalistic calculation. M. D. GOLDFINGER.

The Hodgkin-Huxley formalism is used to calculate the classical anode-break excitatory response, elicited by a 0.1 msec pulse of hyperpolarizing current (200–215 $\mu A/cm^2$). The differential equations are solved by an unmodified Euler numerical integration over increments of time small enough (1–10 μsec) that no variable changes more than 1–2% per iteration. Calculated values of membrane potential, conductance parameters, and derived components (*e.g.*, ionic currents) are plotted in time and phase plane. All calculations are performed under the constraint of membrane voltage spatial uniformity. The anode-break action potential is of normal duration and form in the period from maximum slope of the rising phase to the recovery from the undershoot; variation from cathodally elicited peak-to-peak amplitude is attributable to differences in the ratio of sodium to potassium conductances at a given isopotential level. The anode-break action potential is triggered by a net inward-going ionic current whose majority carrier is a component associated with a constant leakage conductance.

Phase space analysis suggests that the process of excitation via anode-break is smooth and continuous. In the V-m reduced system during the period of post-anode-break conditioning, the V nullcline is lowered; by the time the phase point returns to the resting potential level, the saddle point and resting stable point are eliminated as nullcline intersections, due to the increase in h and the decrease in n. As a result, in the complete four-dimensional system, the resting steady-state point and the quasi-threshold point disappear and the phase point trajectory is observed to be continuous in any V-conductance parameter phase plane. Thus, anodebreak excitation has no threshold event of the usual type and is another example of departure from the all-or-none law.

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Is the redistribution of tosylerginine methyl ester hydrolase activity that follows fertilization of Arbacia punctulata *eggs intimately related to embryogenesis?* ALBERT GROSSMAN, LAIRD CAGAN, MILTON LEVY, WALTER TROLL, STEVEN WECK AND GERALD WEISSMANN.

Unfertilized sea urchin eggs contain an esteroprokese capable of hydrolyzing tosylarginine methyl ester (TAME). The enzyme, which for the time being is referred to as tosylarginine methyl ester hydrolase (TAMEase), has been shown to be associated with readily sedimentable particles ($750 \times g$). Enrichment of these particles by differential centrifugation, followed by discontinuous sucrose gradient centrifugation indicated that most of the TAMEase activity was associated with a particle having a density of about 1.20. Following fertilization (5 and 30 minutes) there was a diminution of TAMEase activity in this density zone of the gradient. A considerable fraction of this decreased activity could be accounted for by a shift of the enzyme to a less dense layer of the gradient. This suggests an alteration of the initial enzyme bound particles. To determine whether redistribution of TAMEase activity results in some fundamental change in the metabolism of the early embryo, a soluble preparation of the enzyme was added to an *in vitro* protein synthesizing system derived from unfertilized eggs. In the one experiment performed (in triplicate) a substantial increase in ^{14}C amino acid uptake into TCA precipitable material was noted. Ribosomes were preincubated with the enzyme for 10 minutes at 37° C; then for an additional 20 minutes after addition of the standard constituents of an *in vitro* protein synthesizing system. No such increase in ^{14}C amino acid uptake was noted when a similar system was incubated at 0° C. Our tentative conclusion is that activation of the unfertilized egg involves unmasking of a latent protein synthesizing capacity by redistribution of this enzyme.

This work was supported by Grants GM 13728 and GM 07213 from the U. S. Public Health Service.

The effect of puromycin on Chaetopterus *development.* PIERRE GUERRIER, PAUL L. KRUPA AND GILLES H. COUSINEAU.

As in other spiralians, normal development in the polycheate annelid *Chaetopterus* involves the production of a plasmatic vegetal polar lobe, which appears to control the formation of the larval apical tuft. As described previously by one of us (P. G.), working with *Sabellaria alveolata,* the cortical responsibile factors may act in an indirect way, inducing some translational process in the cytoplasm.

To test this hypothesis further, *Chaetopterus* embryos developing in 1000 μg/ml sea water were studied. First cleavage was slightly delayed and accompanied by formation of huge protuberances which resulted in some fragmentation. The first cleavage plane was often discernible, however, and the second cleavage spindles developed normally at the time when controls underwent third cleavage. Embryos treated with puromycin for one hour developed into swimming forms when returned to sea water, but they appeared abnormal when compared to controls 24 hours after fertilization. Thus, when studied with borated methylene blue as a vital stain or with the Feulgen reaction, they ranged from uncleaved syncytial forms identical to Lillie's KCl-treated embryos to fully segmented but undifferentiated embryos. Although such forms were ciliated, they always lacked the apical tuft. In contrast, when other control embryos undergoing first or third cleavage were transferred to puromycin for two to five hours and then returned to sea water, larvae always bearing a normal apical tuft were obtained. In these latter controls, polar lobe development was normal, the segmentation schedule was delayed by one cleavage cycle, and the embryos did not differentiate a normal gut.

These data seem at least to confirm that, at first cleavage, a fundamental activating process takes place which is dependent on the formation of a normal vegetative polar lobe.

Studies of the excitation process in nerve with extrinsic fluorescence. M. HALLETT, I. TASAKI, A. SCHNEIDER AND E. CARBONE.

The structure of the squid giant axon membrane and its excitation process have been studied using the technique of extrinsic fluorescence. Analysis of the fluorescence changes with the probe 2-p-toluidinyl-6-naphthalenesulfonate (TNS) utilizing fluorescence polarization reveal that there are two classes of probe molecules which change during excitation. One class is rigidly oriented with the long axis of the molecules parallel (± 15°) to the longitudinal axis of the axon and the other is oriented with the long axis perpendicular to the longitudinal axis. These classes have been demonstrated with action potentials, voltage clamping and hyperpolarizing responses. The presence of both classes are shown by using 2,6-anilinonaphthalenesulfonate and the longitudinally oriented class only is shown with pyronin B. The fluorescence intensity change of TNS with the polarizer and analyzer parallel to the axon is a decrease during the action potential and has been shown to be possibly related to changes in the membrane hydrophobicity. The fluorescence intensity changes of TNS with the polarizer and analyzer perpendicular to the axon (an increase during the action potential) may be due to the changes in calcium concentration at the interface between the inner part of the membrane and the axoplasm. These changes are quite similar to those of the tetracyclines, probes which are very sensitive to membrane bound divalent cations. Chlorotetracycline gave large fluoresecence changes and was the most studied. In a series of tetracycline compounds, the magnitude of the fluorescence change associated with the action potential was similar to the sensitivity of the tetracycline to calcium in the presence of a phospholipid (but not in water). With depolarizing voltage clamp the fluorescence intensity reaches a maximum near the time of the peak inward current. These studies suggest that the calcium concentration decreases at the inner part of the membrane during a membrane hyperpolarization.

Amino acid transport and plasma protein synthesis in toadfish liver in vivo.

AUDREY E. V. HASCHEMEYER AND ROGER PERSELL.

Previous evidence has indicated that changes in rates in the protein synthetic pathway play an important role in the adjustment of metabolism characteristic of temperature acclimation of poikilotherms. In order to determine kinetic parameters for polypeptide chain assembly *in vivo* by tracer methods, it has been necessary to assume that the rate of amino acid uptake by liver cells is fast compared with assembly rates. A method has now been developed for estimation of amino acid transport rates from measurement of the distribution of a labeled L-amino acid and a marker (D-leucine, mannitol or inulin) in liver and in plasma as a function of time after injection via the hepatic portal vein. In toadfish (200-gram) acclimated at 20°, half-times for uptake of L-leucine into the intracellular space of liver were about 10 sec at 20° and 30 sec at 11°. In this study 4 nm of H^3-L-leucine was given in 0.1 ml saline solution containing 40 nm of C^{14}-D-leucine as marker for the extracellular spaces.

The hepatic portal vein injection procedure was also used to follow the appearance of liver-synthesized proteins in blood sampled from the gill arches. The increase of labeled protein in plasma followed an approximately exponential shape with $t_{\frac{1}{2}}$ about 1 hr at 20° and 6 hr at 10°. Lag times for secretion were in the range 0.7–1.1 hr at 20° and 5.0–6.5 hr at 10°. Ratios of labeled protein in plasma and liver were determined at various times and temperatures. In 20°-acclimated toadfish the half-life for turnover of plasma protein was about 9 days at 20° and 16 days at 10°.

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The effect of copper upon collagen fibrillogenesis in Fundulus heteroclitus.

RAYMOND L. HAYES.

Polymerization of molecular collagen during fibrillogenesis is thought to be catalyzed by a copper-enzyme, leading to the formation of an insoluble product. We have investigated the effects of copper deprivation upon collagen metabolism in larvae of the teleost, *Fundulus heteroclitus*. Hatched larvae were grouped according to total body length (millimeters) and transferred to copper-free MBL formula sea water containing 1 μc/ml of 5-H^3-Proline. To control lots, 3–4 μg/ml of cupric sulfate were added; experimental lots were copper-free. Normal copper contents of pre-hatched embryos equal that of sea water until Stage 27. Thereafter, copper levels increase to twice water value at hatching (Stage 34). In post-hatched larvae the copper content reaches a maximum at 5.5 mm but returns to hatching value by 6.5 mm. The average hatching length of these embryos is 5.0 mm; by 1–2 days they reach 6.0 mm and by 7–10 days, 7.0 mm. Collagen metabolism was estimated by recovery of radioactive peptide-bound hydroxyproline, and data calculated as dpm H^3-Hydroxyproline/larva/day. Synthetic activity was indicated by total recovery of label; polymerization (maturation), by recovery of label in the insoluble residue following extraction with 0.5 M buffered citrate, pH 3.8. The pattern of collagen synthesis is altered in copper-deprived animals. Although 5.5 and 6.0 mm experimentals do not differ significantly from controls, the 6.5 and 7.0 mm experimentals do, suggesting a dependency upon environmental copper for synthetic activity. Comparison of radioactivity in insoluble residues indicates an alteration of the pattern of polymerization of collagen with removal of copper. The insoluble pool is elevated in 5.5 and 6.0 mm larvae over control values, but reduced in 6.5 and 7.0 mm larvae, indicating that the latter two require environmental copper for polymerization of collagen. A copper-protein complex has been identified in the skin of adult *Fundulus* using acrylamide gel electrophoresis. This complex migrates as an α-globulin and might mediate the polymerization of collagen in this tissue.

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The effect of hyperbaric oxygen upon the embryonic development of Arbacia punctulata. Paul M. Heidger, Jr., Robert G. Summers, and James A. Miller, Jr.

The observation of Miller *et al.* (1969) that hyperbaric oxygen blocks both embryonic development and succinic dehydrogenase activity in the hydroid, *Tubularia,* prompted an investigation of the effect of hyperbaric oxygen (HBO) upon the embryonic development of *Arbacia* from fertilization to the time of formation of the pluteus larva. Fertilized eggs were incubated in sea water at 16° C in air or in 3 atmospheres absolute pure oxygen in a hyperbaric chamber. Pressure control experiments using 1 atmosphere oxygen and 2 atmospheres nitrogen were conducted to determine that changes observed were due to high pressure oxygen and not merely to high ambient pressure. Animals exposed continuously to HBO for 48 hours were arrested in the gastrula stage; the archenteron was observed to form at 32 hours, but to regress, resulting in an unorganized sphere of cells. If removed from HBO at 48 hours, over 90% of these inhibited embryos proceeded to form normal pluteus larvae within 100 hours following removal. Embryos introduced to HBO following development in air for the first 12 hours after fertilization failed to form plutei by 72 hours, whereas controls did so within 48 hours. Embryos subjected to HBO, following up to 30 hours in air, reached the prism or pluteus stage, but died within 72 hours post-fertilization. Embryos subjected to HBO following 36 hours of development escaped both the inhibitory and lethal effects of HBO and proceeded to form normal plutei by 48 hours.

Hyperbaric oxygen blocks the activity of sulfhydril-containing enzymes, of which succinic dehydrogenase and glucose-6-phosphate dehydrogenase are examples. The high activity of both of these enzymatic activities during the gastrula stage of development in the sea urchin (Backstrom, 1959; Gustafson and Hasselberg, 1951) suggests that depression of the activity of these enzymes by HBO may contribute to the failure of the hyperbaric oxygen treated embryos to complete gastrulation.

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Biogeography of sand beach Gastrotricha from the northeastern United States. William D. Hummon.

A study of 16 beaches, ranging from Long Island, New York, to the New Hampshire border, was completed. Each was analyzed by means of a whole beach transect involving 80–120 faunal samples interspersed from low to high tide-levels and surface to ground-water depths. Ten of the beaches were situated in pairs on five long-shore bars to study the effects of beach exposure on species diversity and population dispersion. Beaches were selected in part to determine whether or not Cape Cod acts as a meiofaunal barrier to intertidal Gastrotricha.

A total of 23 species of Macrodasyida and 19 species of Chaetonotida were found, with 4–20 ($\bar{X} = 11$, s.d. $= 4$) species per beach. The number of species per beach, though not necessarily the number of individuals, tends to decrease with increasing exposure to wave action (4–6 species on the three highest-energy beaches) and to decrease with increasing organic matter (7 species on the most detritus-laden beach). Populations tend to be dispersed more deeply in exposed beaches and more shallowly in beaches having a high organic content than in clean, semi-protected beaches. Individual species were found on 1–16 ($\bar{X} = 4$, s.d. $= 4$) beaches, at the extremes with 15 species found on only one beach, 7 species on two beaches, two species on 15 beaches and one species on all 16 beaches. Of 27 found on two or more beaches, 25 were found both north and south of Cape Cod, indicating that Cape Cod does not act as a significant faunal barrier for this portion of the meiofaunal community.

Aspects of the study were supported by NDEA and NASA Fellowships, by the Systematics-Ecology Program (Marine Biological Laboratory), and by Ohio University.

Jelly coat material of Arbacia *eggs: an electron diffraction study.* SADAYUKI INOUE, GILLES H. COUSINEAU AND PAUL L. KRUPA.

The jelly coat substance from the eggs of the sea urchin *Arbacia punctulata* is known to be elongated molecules of homogeneous material (molecular weight, 300,000). In this study the structure of jelly coat substance has been studied with the technique of electron diffraction.

Arbacia jelly coat was isolated by the treatment of eggs with acidified sea water (pH, 5.0), dialyzed against distilled water (4° C) followed by freeze drying. From 15% aqueous solution thin films of specimen were casted on carbon film-coated grids. Electron diffraction patterns were recorded in a JEM-100B electron microscope with the specimen-plate distances of 200, 400 and 800 mm.

Both fiber and powder diffraction diagrams were obtained and this suggested the presence of crystallites oriented either parallel or at random. The spacings of 23 reflections were calculated. Assuming three major equatorial reflections as 101 (6.40 Å), $10\bar{1}$ (3.71 Å) and 002 (3.19 Å), unit cell dimensions were calculated by the reciprocal lattice method. By using $T = 10.11$ Å as the fiber repeat instead of a rather uncertain value (8.84 Å) estimated from faint 0k0 reflections, a monoclinic cell with $a = 7.46$ Å, $b = 10.11$ Å (chain axis), $c = 7.35$ Å and $\beta = 60.0°$ was obtained. All other reflections seemed to be successfully indexed with these parameters.

According to Vasseur (1952), polyfucose sulfate, the main component of egg jelly of sea urchins, is linear polymer in which each fucose unit has one fucose sulfate as a side chain. It might be possible to assume that in the unit cell described above, two of these molecules ($\frac{1}{4}$ at each corner and 1 at center) pass perpendicularly through *a–c* plane with *b* corresponding to the length of 2 fucose units (4 fucose sulfate/unit cell). The nature of chemical bonding across 101 planes might be bridges of -(sulfate)-Ca-(sulfate)-.

A histological study of the accessory pigment spot of Palaemonetes vulgaris. STEPHEN K. ITAYA AND JOHN C. CORNELL.

The accessory pigment spot in *Palaemonetes vulgaris* is about 0.1 mm in diameter consisting of about 20 ommatidia like structures extending proximally from the compound eye to the dorsal surface of the eyestalk. The ommatidia of the accessory pigment spot show the generalized features of crustacean ommatidia and are about half the length, and proportionately wider than those found in the main compound eye of *P. vulgaris.* In contrast to the main compound eye the cornea of the accessory pigment spot is composed of irregularly shaped facets, below which are the cornea cells. Four crystalline cone cells are located above the cylindrical crystalline cone which tapers proximally and becomes surrounded by pigment. A rhabdome is located below the crystalline cone and nerve fibers appear to connect the ommatidia with the lamina ganglionaris. Preliminary results suggest that the accessory pigment spot may have a controlling effect on the migration of pigments in the main compound eye.

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Inhibition of dogfish retina protein synthesis by near UV light. S. JOHNSON AND S. ZIGMAN.

Former studies have shown that near UV light inhibited total protein synthesis of dogfish (*Mustelus canis*) retinal rods *in vitro* by 50 to 70%, but attempts to ascertain the effect on the specific synthesis of rhodopsin were not done. In other studies, the photoproduct of near UV-irradiation of tryptophan was found to combine with amino groups in many proteins, RNA and DNA. The experiments done here were undertaken to observe inhibitory effects of near UV light and its tryptophan photoproduct on rhodopsin synthesis.

Retinas freshly excised in dim red light were incubated aerobically in non-urea containing elasmobranch Ringer's solutions for 3 hrs at 22° C (12 to 15 per flask). Flasks were kept in total darkness, in incandescent light (*ca.* 200 ft-c. from a 75 w bulb), or under 3000 to 4000 $\mu w/cm^2$ of near (340 to 380 nm) UV light. When the effects of tryptophan photoproducts were to be investigated, 0.1% tryptophan was added to all flasks, and the UV irradiation was begun 24 hrs before addition of the retinas. To all flasks, 2 μc of C^{14}-amino acid mixture were added. Rods were isolated by shaking the retinas in complete Ringer's medium (containing 0.3 M urea) for 15 min, spinning down residual retinal tissue at slowest speed and then packing the rods down at the highest speed in a clinical centrifuge in the refrigerator. Rods were extracted with 1% triton × 100 or emulphogene BC720. The extracts were electrophorsed on 5 or 7.5% polyacrylamide gels with 1% detergent for 3 hrs at 5 mà per tube. Tris-glycine buffer at pH 8.3 was used. Gels were stained with amido schwartz. Others were sliced into 2 mm slices lengthwise, and the radioactivity of the slices was determined after dissolution in 30% H_2O_2.

Different banding patterns were obtained for the rod extracts that were maintained under incandescent light or darkness. The major band (that is, rhodopsin) was found by visualization to remain close to the top of the gel. It contained the greatest radioactivity of all 5 bands obtained. No difference in C^{14} incorporation into rhodopsin was observed in light or dark. Near UV light alone inhibited incorporation by 50%, while the UV tryptophan photoproducts inhibited some 90% of amino acid incorporation.

These results indicate that near UV light is a potent inhibitor of rhodopsin synthesis by acting directly on the rods or by photooxidizing aromatic compounds to inhibitory substances. Retinas exposed to near UV light could be damaged in this way.

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Cryolite: a new technique for observing burrowing in aquatic animals. R. K. JOSEPHSON AND K. W. FLESSA.

The principle difficulty in studying the activities of burrowing organisms is that natural sediments are opaque and the organism disappears from view as it goes beneath the surface. The opacity of natural sediments is due to light absorption and refraction. Light absorption can be minimized by using sediments made from transparent material such as glass particles but light refraction at the particle-water interfaces still limits visibility to a few millimeters at best. Light refraction can be reduced by making sediments from cryolite, a mineral with an index of refraction nearly identical to that of sea water. Sediments made from crushed natural cryolite, obtained as mineral specimens, are nearly transparent and the activities of burrowers can be observed through at least one centimeter of sediment. Although cryolite is used as an insecticide, in our experience it has not proved harmful to marine organisms (clams, polychaetes, anemones) in short term exposures. Cryolite is sparingly soluble in water (2 mM at saturation) and can be crushed easily to produce sediments of any desired particle size.

The relationship of hinge ligaments and adductor muscles in various bivalve mollusks. GEORGE A. KAHLER, JR. AND FRANK M. FISHER, JR.

The relationship between the strength of hinge ligaments and the quantity of muscle opposing the ligaments was examined for the swimming bivalves, *Placopecten magellanicus* (Gmelin) and *Aequipecten irradians* (Lamarck); the burrowing bivalves, *Ensis directus* (Conrad) and *Mya arenaria* L.; and the attached forms, *Mytilus edulis* L. and *Crassostrea virginica* (Gmelin).

The strength of the ligament was measured as the applied moment in gram millimeters at which the valves were capable of beginning to gape, or the "opening moment." The quantity of muscle opposing the ligament was expressed as the product of the distance from the center of the muscle mass to the center of the hinge and the dry weight of the total muscle, the "muscle mass moment." The ratio of "opening moment" to "muscle mass moment," H/M, was calculated for each of the bivalves examined.

The ratio was smallest for *P. magellanicus* and *A. irradians*. The burrowing bivalves, *E. directus* and *M. arenaria*, had smaller H/M values than *M. edulis*, but significantly larger values than *C. virginica*. The fast and slow portions of the adductor were separated in *A. irradians* and *P. magellanicus;* the H/M value was then recalculated on a dry weight of fast muscle basis. The new value for *A. irradians* still shows an H/M value less than $\frac{1}{10}$ that of the value for *M. edulis* which was calculated from the total dry weight of the adductors.

P. magellanicus and *A. irradians* possess more muscle in relation to hinge strength probably as an adaptation for the quick and powerful closing of the valves during the power stroke of swimming. The relatively low H/M values for burrowers reflect the use of mechanisms other than the hinge to open the valves.

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Ultrastructural demonstration of cytochrome oxidase activity in mitochondria of digenetic trematode larvae. PAUL L. KRUPA, BURTON J. BOGITSH AND GILLES H. COUSINEAU.

Localization of pigment resulting from the oxidation of diaminobenzidine (DAB) was studied with the electron microscope in mitochondria of *Schistosoma mansoni* sporocysts and cercariae. Tissues from infected snails (*Biomphalaria glabrata*) were fixed in 2% distilled glutaraldehyde, cut at 65 μ on a tissue chopper, incubated for 1 hour at 37° C in the DAB medium (pH 7.4) of Seligman *et al.* (1968), and processed for electron microscopy. All mitochondria were reactive, whether they were located in the tegument or subtegumental regions of the sporocyst body wall or of intrasporocyst cercariae. Mitochondrial enzyme reaction product appeared within the intracristal space, along the outer surface of the inner membrane, and within the compartment between the inner and outer mitochondrial membranes. In parasites incubated with DAB and KCN no deposits appeared in any mitochondria.

Other tissue sections were incubated for 1 hour at 37° C in the DAB medium of Novikoff and Goldfischer (1969) for the detection of catalase or peroxidase activity in peroxisomes (microbodies). Controls consisted of the complete medium with KCN to inhibit mitochondrial enzyme activity, or with aminotriazole (AT) to inhibit catalase. At this writing no observations had been made on specimens incubated with DAB at pH 9.0, but in those incubated with DAB and AT an intense reaction product was found in all sporocyst and cercarial mitochondria. In specimens incubated with DAB and KCN mitochondrial enzyme activity was blocked. Uniformly dense bodies were encountered in the body wall of sporocysts incubated with DAB and KCN, but more observations are needed to determine if these are peroxisomes.

The mitochondrial oxidation of DAB at pH 7.4 and 9.0 was presumably due to cytochrome oxidase reactions. The demonstrability of this enzyme activity in all mitochondria of both larval stages contrasts with the results of a previous study by two of us (P. L. K. and B. J. B.) where adenosinetriphosphatase activity was detectable in only some mitochondria of *S. mansoni* intrasporocyst cercariae fixed in formaldehyde.

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Sponge aggregation II: Immunologic studies on cell-cell interaction sites. WILLIAM J. KUHNS AND MAX M. BURGER.

Agglutinins from plant as well as animal sources have shown to be useful in efforts to identify the chemical nature of specific macromolecules on surface membranes. Such a direct approach seemed not to be feasible for the identification of the surface sites involved in the aggregation of sponge cells since none of the plant agglutinins tested was able to bring about agglutination of any of the three sponge species tested.

The following immunological approach led however to a simple and generally useful screening assay. A series of plant agglutinins were preincubated with sponge cell aggregation factor that was released during chemical dissociation from sponge cell surfaces. Subsequent addition of erythrocytes (human, baboon, etc.) revealed those agglutinins which were not able to agglutinate the erythrocytes anymore and therefore might be recognizing sites or neighboring sites on the 100 S aggregation factor particle which are involved in the cellular aggregation.

Out of 14 agglutinins examined only 4 were found to be inhibitory (*Lens culinaris, Pisum sativum, Vicia faba* and *Conavalia ensiformis*) in such an assay with *Microciona prolifera* factor but not with two other species of sponges (*Cliona celata* of *Haliclona occulata*). The same four and no other agglutinins also inhibited the aggregation of chemically dissociated *Microciona* cells by their corresponding factors.

Since α-methyl glucopyranose and α-methyl mannopyranose are strong inhibitors of human erythrocyte agglutination by *Conavalia, Pisum* and *Vicia* agglutinins, more so than N-acetylglycosamine, these carbohydrates were tested as possible inhibitors of *Microciona* cell aggregation. α-methyl glucose and α-methyl mannose inhibited indeed at 2×10^{-3} M, N-acetylglucosamine inhibited poorly at 2×10^{-2} M while N-acetylgalactosamine, glucose, galactose and a series of carbohydrates known to occur in cell surface glycoprotein and glycolipids did not inhibit at 2×10^{-2} M as was predictable.

Plant agglutinins may become useful tools to identify surface macromolecules involved in various cellular interactions with biological significance.

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RNA synthesis during oocyte maturation in Asterias forbesi. MICHAEL J. LAMARCA, ELIZABETH L. SHIPPEE AND ALLEN W. SCHUETZ.

The pattern of RNA synthesis in oocytes during the process of nuclear maturation was investigated. After incubating ovarian segments in filtered sea water containing antibiotic, ^{3}H-uridine (150 μc/ml) and 1-methyl adenine (0.25 μg/ml), synchronously maturing oocytes were collected free of follicles. RNA was extracted from shed oocytes with cold phenol and analyzed by sucrose gradient centrifugation. Oocytes incubated for periods varying from 45 minutes to 7½ hrs after 1-methyl adenine exposure all display the same pattern of radioactivity: a high peak of low molecular weight (4–5S) RNA, a lower broad peak of intermediate weight (15–19S) RNA, and no significant incorporation in the 26S region. In contrast, oocytes incubated 5½ hrs prior to induction of maturation demonstrate synthesis of large quantities of 26S and 18S rRNA.

Inhibitors of RNA synthesis were used to study the nature of the radioactive RNA. RNA was extracted from maturing oocytes incubated for 2 hrs in sea water containing ^{3}H-uridine (150 μc/ml) and actinomycin D (10 μg/ml) or ethidium bromide (10 μg/ml). Actinomycin D reduced synthesis of RNA by 53% and ethidium bromide by 82% which suggests the incorporated radioactivity in the controls is the result of DNA transcription. Identical doses of either inhibitor does not prevent germinal vesicle breakdown or fertilization indicating concurrent RNA synthesis is not necessary for ovulation, maturation or activation. Ethidium bromide in low concentration (2.0 μg/ml) blocked synthesis of virtually all of the 15–19S RNA and this suggested a mitochondrial origin of this class of RNA. Mitochondria were isolated from oocytes incubated in ^{3}H-uridine during maturation following homogenization in cold Tris-EDTA-sucrose buffer (pH 8.0) and centrifugation at 10,000 *g*. Analysis of RNA from the mitochondrial fraction displays a very low 4–5S peak of radioactive RNA and the broad peak in the 15–19S region. These observations suggest at least part of the RNA synthesis during maturation occurs in mitochondria.

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Media for the enumeration and selective isolation of salt marsh epiphytic algae, bacteria, protozoa and micrometazoan herbivores from the community. JOHN J. LEE, JOHN H. TIETJEN, AND EILEEN KENNEDY.

Nutritional studies of salt marsh epiphytic diatoms and chlorophytes led to the design of a series of sea water based and artificial media which, in combination, were extremely useful in characterizing succession in the algal community and in the isolation of over 56 species of diatoms during the summer. This was 4 times the number of species isolated on erdschreiber medium alone. Many species of algae grew in unenriched sea water from the same field station. Media varied greatly in their selectivity for diatom species; enriched synthetic media were more stimulatory than enriched sea water media. The largest number of species were isolated on media enriched with: nitrate; thiamine, biotin, and B_{12}; soil extract; mannitol; a vitamin mixture; or an acetone extract of *Enteromorpha intestinalis*. A key for the identification of diatom colony types was developed. Diatoms fixed and preserved at the time of collection are being studied to estimate the efficacy of the media. Preliminary estimates suggest that approximately 60% of the most numerous (> 3%) diatoms in the community were isolated on one or more of these media.

As was hoped the new media opened the way for the culture and laboratory study of many hitherto unisolated salt marsh micrometazoa and microorganisms. Of particular interest to us are the nematodes isolated from Sippewissett marsh. The nematodes presently growing in agnotobiotic culture are *Axonolaimus* sp., *Monhystera denticulata, Chromodora germanica, Desmodora* sp., *Euchromodora* sp., *Oncholaimus* sp., and *Theristus* sp. Preliminary physiological-ecological studies of *Monhystera denticulata* have begun. At 26‰ salinity and 25° C its life cycle is completed in 10–14 days; at 15° C it is lengthened to 16–20 days. At salinities above and below 26‰ life cycles are lengthened.

Different aspects of the study were supported by NSF GB 19245 and AEC contract AT (30–1) 3995.

Intracellular injection of Ca^{++}-EGTA solutions in Limulus *ventral photoreceptors.*

JOHN E. LISMAN AND JOEL E. BROWN.

The primary mechanisms of light-adaptation is a reduction in the size of the quantum bumps which summate to form the receptor potential. The initial large transient phase of the light response is due to a delay in the reduction of bump size. Bump size can be reduced by intracellular iontophoresis of calcium. We propose that a light-activated increase in intracellular calcium accounts for light-adaptation in *Limulus* ventral photoreceptors. To test this we attempted to stabilize the intracellular calcium concentration by intracellular pressure injection of EGTA solutions. 0.1 M EGTA (no calcium added) caused the receptor potential to rise to reversal potential for the duration of a bright light. Such large responses could not be produced by larger injections of 0.1 M mannitol ($S^{35}O_4$ marker). Injection of 0.1 M EGTA adjusted to 10^{-6} M free calcium lead to not change in the size of the steady-phase of the light response for stimuli 3.7 log units above threshold. Injection of 0.1 M EGTA with 10^{-5} M free calcium lead to no change in the steady-phase amplitude for stimuli 6.0 log units above threshold. In either case, the responses elicited by dimmer stimuli were smaller following injection whereas the responses to brighter stimuli were larger following injection. After a sufficient injection of Ca^{++}-EGTA solution the light-activated current had only a steady phase whose amplitude varied linearly with light intensity. These results are consistent with the hypothesis that the size of the quantum bumps is fixed by the action of the Ca^{++}-buffer. However, linearization of the intensity response curve in part may be artifactual because it can also be produced by massive injection of 0.1 M mannitol.

Supported in part by NIH grants EY-00151, EY-00312 and EY-00377.

Some effects of colchicine on regeneration in Tubularia.

ANTHONY LIUZZI AND DAVID A. SNYDER.

Colchicine was used to determine the possible morphogenetic role of microtubules in regenerating *Tubularia* stems. Experiments were done using freshly cut stem segments approximately 7 mm long just below the hydranth from clean healthy colonies of *Tubularia*. Groups of 20 stems were maintained in finger bowls containing filtered sea water with and without various concentrations of colchicine.

No difference in wound healing was observed between controls and stems placed immediately after cutting and maintained in concentrations of colchicine ranging from 2.5×10^{-7} to 2.5×10^{-3} M. At concentrations of 2.5×10^{-4} M and greater, all stems were completely arrested at the morphological state just following wound healing. At concentrations of 2.5×10^{-5} M and below there was no discernible difference in regeneration between the treated and control groups.

Immediately after cutting, groups of stems were maintained in 2.5×10^{-4} M colchicine for various times ranging from 1 hour to 73 hours, then washed and placed in colchicine-free sea water. In all cases, stems fully regenerated after removal from colchicine. However, the regeneration rate was observed to increase with the maintenance time in colchicine. In this experiment, controls completely regenerated hydranths within 92 hours.

Stems cut and maintained in filtered sea water were placed in colchicine sea water at various times after cutting. Only those stems introduced to colchicine before wound healing were inhibited from regenerating hydranths. All stems introduced to colchicine thereafter continued to regenerate at the same rate as the controls and with no detectable morphological differences.

Uptake of pulsed tritiated colchicine in the tissue mass within the perisarc was identical in control and colchicine maintained stems.

It is planned to extend the study of colchicine inhibition of microtubules in *Tubularia* using electron microscopy, isotope scanning and procedures for examining DNA and protein metabolism. It may thereby be possible to determine the specific role of microtubules in this morphogenetic system.

Modulation of the burst discharge rate of the lobster cardiac ganglion by an electrogenic Na⁺ pump. DAVID R. LIVENGOOD, TERRENCE L. PENCEK AND KIYOSHI KUSANO.

The heart rate of many, if not all, Crustacea is controlled by the rhythmic discharge of a cardiac ganglion. In the American lobster the cardiac ganglion discharge rate can be modulated by an electrogenic Na^+ pump. Intracellular recording techniques indicate that an electrogenic Na^+ pump mechanism exists in pacemaker cells as well as the follower cells of at least three species of Crustacea: the American lobster, the spiny lobster and the mantis shrimp. Perfusion experiments indicate that in the American lobster the modulation of burst discharge frequency takes place predominantly at the level of the pacemaker cells in the intact ganglion. The heart beat of three species of Crustacea, American lobster, blue crab, and mantis shrimp, was shown to be modulated by factors which affect the electrogenic Na^+ pump. Factors that slow the pump rate tend to increase the beat frequency while factors that increase the pump rate tend to slow the beat frequency. The modulation of the burst discharge frequency, and consequently the heart rate, seems to be due to control of the membrane potential of the pacemaker area.

This work was supported in part by a research grant from the National Institute of Neurological Diseases and Stroke, U. S. Public Health Service (N.S.-09618) and training grant (M.H.-10695). One of us (D. R. L.) was supported by a Grass Foundation Fellowship at the Marine Biological Laboratory, Woods Hole, Massachusetts.

Pharmacology and reflex responsiveness of the heart of the giant garden slug, Limax maximus. ALEX R. MACKAY AND ALAN GELPERIN.

The heart of the terrestrial pulmonate *Limax maximus* comprises an auricle and a ventricle. Each chamber is innervated by a branch of the visceral nerve which issues directly from the visceral ganglion. Heart preparations at different degrees of isolation from the central nervous system, including intact preparations, have been used to examine the mechanism of cardioregulation and its involvement in behavior. Acetylcholine (10^{-8} M) exerts negative inotropic and chronotropic effects upon the isolated heart, eventually producing diastolic arrest (10^{-5} M). Serotonin (5-HT) exerts positive inotropic and negative chronotropic effects at low concentration (10^{-7} M). At higher concentrations (10^{-5} M) the chronotropism becomes positive and the heart beats in bursts. Norepinephrine has a positive inotropic action. Benzoquinonium blocks the effects of ACh, and methysergide and BOL block the effects of 5-HT. Suction electrode recording from the ventricle reveals that each contraction is preceded by a fast positive spike superimposed upon a slow depolarizing pacemaker wave. Suction electrode stimulation over cell bodies in the suboesophageal ganglia has located three cardioregulatory regions, two inhibitory and one excitatory. Preliminary intracellular studies located a tonically active cell which exerts strong cardioexcitation. Study of the heart rhythm in the intact animal has shown that tactile stimulation of the tail evokes cardioexcitation accompanied by tentacle extension and movement away from the stimulus. Tactile stimulation of the head elicits cardiac inhibition or arrest in extreme cases. This reacton is accompanied by protective withdrawal of the tentacles and head beneath the mantle flap, but may occur in an animal which has already withdrawn. Injection of eserine into the haemocoel of the intact animal greatly potentiates the cardiac inhibition produced by head stimulation. The pharmacology of the *Limax* heart is similar to that of *Helix* and *Mercenaria,* in which the excitation is serotoninergic and the inhibition cholinergic. Cardiac excitation and inhibiton are clearly elements of simple behaviors resulting from stimulation of different receptive fields.

This work was supported by National Science Foundation Grants GB 20762 and GY 8773.

In vivo *radioactive labeling of mitochondrial DNA in* Arbacia punctulata *oocytes.* LLOYD MATSUMOTO AND LAJOS PIKÓ.

The synthesis of mitochondrial DNA and its radioactive labeling during oogenesis have not been shown conclusively in sea urchins. We injected 200 μc of ^{3}H-thymidine (20 c/mM) into each of three *A. punctulata* females, following force-shedding with KCl. Two weeks later the same females shed a total of about 3 million eggs. Mitochondria were isolated according to Ozaki and Whiteley (1970). The final purification step in a sucrose density gradient yielded two mitochondrial bands: M1, density 1.19 and M2, density 1.17. Electron microscopy showed that M1 contained possibly damaged mitochondria and some cytoplasmic debris while M2 contained essentially pure mitochondria. Mitochondria were lysed with SDS-EDTA and their DNA was isolated by equilibrium centrifugation in a CsCl-ethidium bromide (EB) density gradient. The M1 and M2 fractions each gave two DNA bands, about 5 mm apart, visible in UV light. About 50 λ fractions were collected on paper discs, washed with cold TCA and counted. Two peaks of radioactivity, coinciding with the two DNA bands, were obtained. The M1 and M2 preparations gave a combined total of 10,540 cpm: 70–80% of the radioactivity was in the lower DNA band (covalently closed circular DNA) and 20–30% in the upper band (linear and nicked circular DNA). These results show that mitochondrial DNA is synthesized and can be labeled radioactively during oogenesis. Fertilized *A. punctulata* eggs (0.5 ml packed eggs in 2.5 ml sea water) were also cultured in the presence of 100 μc/ml ^{3}H-thymidine (6.7 c/mM) for 6 hr (morula). The mitochondria were purified as described above and were treated with DNase to remove contaminating nuclear DNA. Centrifugation in a CsCl-EB gradient gave two DNA bands (presumably representing nicked and closed circular mitochondrial DNA) but isotope counting did not detect label in either of these bands. This observation shows that mitochondrial DNA does not replicate during cleavage in *A. punctulata* eggs; a similar finding was made in *L. pictus* eggs (Piko, 1969).

This study was supported by NIH grant 5-T01-HD00026-10 to the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory.

Genic polymorphism and population dynamics in Mytilus edulis. ROGER MILKMAN.

Observations of striking uniformity in the frequencies of 3 common alleles at the LAP locus in *Mytilus* confirm 1970 findings and extend the uniform region west to Narragansett Bay. The frequencies appear habitat- and size-independent in this region. Cape Cod Bay populations are again much lower in the frequency of the slow allele; again they differ somewhat from one another. Intermediate regions contain populations, notably in the Cape Cod Canal, that show radical size-dependent variations in frequencies; these move upward through size classes with time. These populations must have mixed origins. It is concluded that the uniform population contributes spat to the Canal region until early- or mid-August, after which a more northerly population makes the major input. A study of the literature on current patterns supports this conclusion.

This technique permits the study of *population circuitry,* a term descriptive of the apparent "round robin" nature of population maintenance in certain coastal areas. It appears possible that many *Mytilus* populations receive major input neither from their own spawn nor from a representative sample of nearby populations, but rather that certain larval pathways connect some populations in ultimately closed circuits and intersect in other populations.

Growth rates can be followed in heterogeneous populations; it is proposed that individuals in the Canal population grow an average of at least 22 mm in their first year. Observations in years to come should test the uniformity of the input pattern, which may vary as currents do from year to year. In regions where populations of mixed origins occur, we may expect LAP allele frequencies to vary with habitat, since they provide a natural tag, reflecting complete genomes associated with one area of origin or another.

In vitro *fertilization and capacitation-like interaction in the hydroid* Campanularia flexuosa. Michael G. O'Rand.

Fertilization in *C. flexuosa* is preceded by sperm attraction to the female gonangium and sperm migration to the eggs. Spermatozoa migrate from the distal edge of the mature female gonangium funnel along the inner funnel wall into numerous passages which lead to the eggs. Shortly after gonangium maturation, these passages normally contain spermatozoa which await oocyte maturation. The spermatozoa remain capable of fertilizing eggs for at least four days. Eggs may be removed from a gonangium as a single packet but surrounded by epithelial cells and still attached to the blastostyle. Eggs present in such packets continue to mature *in vitro.* Upon completion of maturation they are fertilized by sperm in the packets. Egg packets taken from gonangia cultured in the laboratory isolated from male gonangia do not contain spermatozoa, but ova in these packets may be fertilized by the addition of spermatozoa. Fertilization of ova fails to occur in packets containing sperm if the packets are exposed to calcium and magnesium free sea water, pronase, phospholipase C or trypsin prior to egg maturation. Eggs in trypsin (0.75%) treated packets may be fertilized by addition of sperm which have been exposed to female epithelial cells. Exposure of sperm to trypsin treated female epithelial cells will not restore fertilizability. Therefore, a female epithelial cell-spermatozoan interaction is necessary for fertilization. This phenomenon is analogous to the necessary conditioning of amphibian sperm and capacitation of mammalian sperm prior to fertilization.

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Light adaptation, dark adaptation, and pigment migration in the eye of the living squid. Alan L. Pearlman and Nigel W. Daw.

The retina of the squid (*Lotigo pealei*) contains only receptors and the synaptic terminals of efferents from the optic lobe; it therefore offers interesting possibilities for the study of adaptive processes in photo receptors. Recordings of axon spikes or the field response were made from the back of the eye in the restrained, unanesthetized squid. The cornea and iris were removed; the beams of a background and test spot projector were combined, and the field stop focused on the retina producing a stimulus spot of about 50°. The light source was focused on the pupil, to give a Maxwellian view system. An increment threshold curve obtained in this situation has a slope of 1 over a range of 4–5 log units, and shows evidence of saturation at higher levels. During light adaptation with backgrounds that are dim or of moderate intensity (up to 10^{11}–10^{12} quanta/cm^2/sec on the retina) increment thresholds are initially high, but fall 1–1.5 log units in the first 90 sec. Subsequent dark adaptation is rapid (5–10 min) and complete. After exposure to backgrounds that are estimated to be only slightly more intense than the squid might normally encounter, dark adaptation is incomplete, often terminating 2–3 log units above pre-exposure absolute threshold. Such failures of dark adaptation persist for as long as any preparation has been followed (5 hours). Dim or moderate backgrounds cause the melanin screening pigment to move out to the tips of the receptors, and then return to the pigment layer at the outer segment bases. After backgrounds that cause an irreversible elevation in absolute threshold, the screening pigment remains at the tips of the receptors indefinitely.

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Protein initiation in developing sea urchin eggs: A preliminary report. Carmencita G. Pilapil, Paul L. Krupa, Sadayuki Inoue, Enriche C. Preddie, Pierre Guerrier and Gilles H. Cousineau.

An *in vitro* assay of amino acid incorporation into protein contained the following: 70 mM NH$_4$Cl, 12 mM Mg-acetate, 1.2 mM ATP, 0.3 mM GTP, 18 mM creatine phosphate, 8 μg creatine phosphokinase, 60 mM tris-HCl (pH 7.8), 16 mM mercaptoethanol, 3.3 mg tRNA purified from either *E. coli* or developing sea urchin eggs, 3–5 O.D. of ribosomes, 200 gammas of protein initiation factors (35% and 80% ammonium sulfate saturation), 200 O.D. of mRNA in the case of R17 and 100 μg in that of poly U and poly A, 200 gammas protein from the 140,000 *g* supernatant and 2.5 mM of the 20 amino acids with either lysine or phenylalanine labeled to a specific activity of 10 μc/μM. The total volume of each assay was 125 lambdas, and each reaction tube was incubated at 37° C for 30 min with constant agitation. Reactions were stopped by the addition of 5% TCA (final concentration) and the material hydrolyzed at 90° C for 15 min. The precipitates were then recovered on Millipore filters, washed thrice with 5% TCA, and finally analyzed with a Packard Liquid Scintillation Counter. The results obtained from these experiments showed that poly U, in the case of *E. coli*, is twice more active in the incorporation assay than the R17 mRNA. Further, it was seen that not only does the activity of ribosomes increase from unfertilized eggs to blastulae embryos (with poly U), but that washed and frozen ribosomes are much more efficient than intact ribosomes, as obtained from these different stages and tested with poly U. Lastly, it was observed that when poly A was used as template the values obtained in the incorporation assays were much lower. It is suggested that the activities reported in these experiments are a major expression of endogenous mRNAs, when these are protected by the presence of exogenous pyrimidines. A test of this hypothesis is presently underway.

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Localization of light producing cells in adult Mnemiopsis. GEO. T. REYNOLDS, GARY FREEMAN, AND J. R. MILCH.

The localization of the light producing cells in the meridional canals of the Ctenophore *Mnemiopsis* has been accomplished by means of an image intensifier-microscope combination at high gain and high magnification. Recording on magnetic tape using a plumbicon vidicon to observe the image intensifier output permits a time resolution of 16 milliseconds and thereby prevents loss of spatial resolution due to the motion of the specimen during the luminescence.

The light producing cells are in the meridional canal, and are asymmetrically located with respect to the axis of the canal as viewed perpendicular to the plane of the comb plates. By observing adjacent pairs of long and short canals, and making use of the fact that the gonads are imaged in each pair, it has been possible to localize the light producing cells to that side of the canal that contains the testis.

By investigating the detailed distribution of the light producing cells it is clear that they are not identical with the testis and in fact extend into regions near the comb plate attachment where there are no gonads. A group of cells can be identified in stained sections that have a distribution consistent with that of the light producing cells. This cell population is located below the testicular tissue on the side wall of the meridional canal. They are separated from the lumen of the canal by a layer of gastrointestinal cells. The cytoplasm of these cells stains lightly with basophylic dyes. The nucleus contains a small nucleolus, the chromatin is condensed.

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Circular dichroism and the revised structure of d-tubocurarine. JAMES T. ROBERTS AND RICHARD J. KITZ.

In order to explore the influence of the —OH groups of d-tubocurarine on its binding ability and to correlate this with the recently revised structure of this compound, the following experiments were performed. The circular dichroism (CD) of d-tubocurarine was measured on a Carey model 61 recording spectropolarimeter. The CD spectrum showed a strong negative maximum at 198 nanometers (nm) and a positive maximum at 212 nm. Four weaker positive maxima at 266, 273, 280, and 289 nm were noted. As the pH was altered from 7.1 to 8.8, the maximum at 280 nm increased and a new negative maximum at 302 nm occurred. If these two maxima are followed as a function of pH, apparent pKa's of 8.75 and 9.1 are associated with the bands at 280 and 302 nm, respectively.

When d-tubocurarine was methylated exclusively at the —OH groups with diazomethane (in contrast to methyl halide which would react with the one tertiary amine group now found to exist in the revised structure as well as with the —OH groups) the bands at 280 and 302 nm no longer changed with changing pH. From this evidence we may assign the 280 and 302 mu ellipticity to the —OH groups and not to the tertiary amine.

When d-tubocurarine is bound to the model peptide polyglutamic acid at pH 7.4 in 0.015 M phosphate buffer, there is a decrease in the maximum at 280 but not at 302 nm. We consider this to be evidence that the 280 nm maximum may be associated with the —OH group located on the isoquinoline ring, and closest to the quaternary ammonium group. Both the binding and the CD spectrum appear to be influenced by the dipole resulting from the close association of the —OH and quaternary ammonium groups.

Supported by NIH grant GM-15904.

Preliminary studies on brain implants and sex change in Crepidula fornicata *(L.).* W. D. Russell-Hunter, Martyn L. Apley and John L. Banner, III.

Early studies on *Crepidula*, notably by W. R. Coe and H. N. Gould, demonstrated that the usual protandric sequence of immature → male → transitional → female (in time, size and stack position) is determined by a complex interaction of both physiological and abiotic factors.

Our experiments were based on the premises that: (a) suitable isolated males maintained free of female pheromone show an accelerated sex change; (b) some system is integrating the various factors involved in sex change; (c) attempts by earlier workers to produce sex change by injections had failed; and (d) there are neurosecretory cells in the cerebropleural-pedal complex of ganglia (or "brain").

Experimental and control limpets were isolated in individual cells and penis condition was assessed on a 1–9 scale (immature, functional, regressing). Most experimentals (large males) received a brain freshly removed from a *functional* male donor (4% post-operational mortality in 166 implants). In a series of single implant experiments with male brains the onset of sex change was delayed by an average of 4.1 days (30.2 days for operated animals, 26.1 days for controls). Experiments with implanted female brains, and with aqueous and oil extracts of ripe female gonads were inconclusive. The most significant results were obtained from recipients of twelve successive implants of male brains at five-day intervals. Of eleven limpets in this series, six showed gradual regression of the penis to the immature state in a period of 30–35 days, and five remained functional males for over 60 days.

Further experimental work is hampered by the length of time required to recognize sex change, by the almost certainly polygenic basis of sexuality, and by seasonal shifts in reproductive condition. However, penis condition appears to be a valid indicator of gonad function. As regards experimental work on neuroendocrine controls of reproduction, it is clear that these molluscan studies are at a level of sophistication reached by insect and crustacean physiologists about 25 years ago.

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Postfertilization dark recovery of U.V.—irradiated Arbacia *sperm.* Ronald C. Rustad and Patricia M. Failla.

Exposure of *Arbacia* eggs or sperm to either ionizing or U. V. radiation induces a delay of cell division. Eggs and sperm are approximately equally sensitive to γ-radiation, but the sperm are 10 to 15 times more sensitive than the eggs to U. V. radiation. Eggs exhibit a time-dependent prefertilization recovery from γ-ray-induced mitotic delay, but not from U. V.-induced mitotic delay. The pattern of increase in sensitivity to γ-radiation early in the first mitotic cycle suggests a continued postfertilization recovery. In contrast, fertilized eggs exhibit a period of uniform sensitivity to U. V.-induced mitotic delay suggesting no recovery. Thus, either the eggs may lack a repair enzyme which recognizes U. V.-induced lesions, or the primary target may be the cytoplasm of the egg, which shields a more sensitive nuclear or perinuclear target.

The anticipated postfertilization recovery from γ-ray-induced damage to both eggs and sperm has been demonstrated when the mitotic cycle is interrupted by anoxia caused by bubbling N_2 through the sea water. In the present experiments, we have applied this technique to study the postfertilization recovery from damage to sperm induced by either U. V.- or γ-radiation.

The rate of recovery from U. V.-induced mitotic delay appears to be dose-dependent and often smaller than the rate of recovery from a similar degree of γ-ray-induced delay.

In view of the apparent lack of repair of U. V. damage to either unfertilized or fertilized eggs, the present demonstration of postfertilization recovery from U. V. damage to sperm may indicate that the U. V. targets in the egg are cytoplasmic.

This work was supported by contracts with the U. S. Atomic Energy Commission.

Bioluminescence of scintillons deposited on electron microscope grids viewed by image intensification. RUTH SCHMITTER, J. W. HASTINGS, GEORGE T. REYNOLDS, AND J. R. MILCH.

The experiments reported here were concerned with the identification of the scintillon, the bioluminescent particle which occurs in extracts of *Gonyaulax polyedra,* studied here, and other dinoflagellates. The particles, which may be purified by density gradient centrifugation ($\rho = 1.23$ g ml^{-1}), emit a brief (0.1 sec) flash when the pH is lowered from 8 to 5.7. In these experiments, purified scintillons were fixed by centrifugation in a Sorvall SU particle-counting rotor (8000 rpm for 20 min) onto Index electron microscope grids previously coated with a film of parlodion and gelatin. The grids were attached to a microscope slide by double-sticky tape and viewed by the image intensifier coupled with a light microscope. Images of each grid, obtained by rear view illumination, were also recorded.

When the attached scintillons were flushed with 0.2 M citrate buffer (pH 5.2), emission from discrete spots was seen and recorded; the emission from individual spots was estimated to be of the order of 10^3 to 10^4 quanta. The scintillons responsible did not detach during this procedure, for a second flash could be obtained by readjusting the pH, adding the low molecular weight substrate, *Gonyaulax* luciferin, and then again lowering the pH. The grids were therefore prepared for electron microscopy in order to identify the structures present at the grid coordinates from which light flashes were recorded.

The study was supported in part by NSF and by the AEC Division of Biology and Medicine.

A natural experiment using deep-sea invertebrates to test the hypothesis that genetic homozygosity is proportional to environmental stability. THOMAS J. M. SCHOPF AND JAMES L. GOOCH.

Considerable data from classical genetics indicates that ". . . the role of polymorphic systems as an evolutionary mode is dependent upon the greater survival ability of polymorphic populations in an ever-changing environment" (Lewontin, 1958). The deep-sea is universally considered an environment in which almost all ecologic parameters are spatially and temporally uniform, stable and predictable, relative to intertidal and terrestrial environments. Thus the deep-sea has recently been hypothesized to be inhabited by species exhibiting much less genetic variability than species from less stable regions.

Using standard procedures, 10–12 individuals of each of 4 species (*Ophiomuseum lymani, Psolis* sp., an echinoid and a sipunculid) were dredged from 2000 m and were studied by polyacrylamide and starch gel electrophoresis using histochemical staining for a variety of enzymes and general protein. In sum, 12 of the 25 clearly identified loci (48 per cent) are polymorphic (P) and 13 monomorphic (M): ophiuroid (Esterase, M, P; PGI, P; Dipeptidase, M; LDH, M; XDH, M; TO, P; GP, M, P); holothurian (Esterase, M, P; PGI, M, P; Dipeptidase, P; LDH, M; MDH, P); echinoid (Esterase, M; Dipeptidase, P, P; MDH, P; TO, M); sipunculid (Esterase, M, M, P; PGI, M).

This high degree of genetic variability (for this sample size) is the same as occurs in nearly all species obtained from "more variable" environments. Thus, if our material is typical of deep-sea species, then the environment : organism relationship is no different in the deep-sea than in other environments, with respect to genetic variability.

These results corroborate and extend the initial discovery by Professor Roger Doyle, Dalhousie University, based on the same species of ophiuroid from the continental slope off North Carolina (personal communication). We are grateful to Dr. Fred Grasle for providing ship time to Schopf. Supported by the Block Fund, University of Chicago.

Biochemical population genetics of fiddler crabs (Uca). ROBERT K. SELANDER, WALTER E. JOHNSON, AND JOHN C. AVISE.

An electrophoretic analysis of variation in proteins encoded by 8 genetic loci in 2800 fiddler crabs (*Uca*) from 35 localities on the Atlantic and Gulf coasts yielded the following major conclusions. A color "variant" previously considered a morph of *U. pugilator* represents an undescribed species occurring sympatrically with *U. pugilator* near Panacea, Florida, and also extending west to Texas. The two sibling species differ not only in type of hemocyanin (as previously demonstrated by Fielder, Rao, and Fingerman) but also in allelic composition at the *Pgi-1*, Esterase-1, and Esterase-2 loci. Frequencies of four alleles at the *Pgi-1* locus in *U. pugilator* are uniform throughout the range of the species from Cape Cod to Florida, but allele frequencies at the *Est-1* locus are markedly variable regionally.

Genetic analysis confirms a previous conclusion, based on behavioral evidence, that populations on the Gulf coast assigned to *U. pugnax* represent two species, neither of which is closely similar to *U. pugnax* of the Atlantic coast. In *U. pugnax,* allele frequencies at the *Pgi-1* and *Pgm-1* loci are essentially homogeneous in 20 populations sampled on Cape Cod and in three populations on the coast of the Carolinas. However, allele frequencies at a third polymorphic locus (*Got-2*) vary geographically: the *S* allele occurs at frequencies of 0.32 to 0.40 on the eastern part of Cape Cod and 0.09 to 0.19 in Buzzards Bay and along the south side of the Cape, but is absent in three samples from North and South Carolina.

The heterogeneity in interlocality allele frequency variances among loci in *U. pugnax* and *U. pugilator* is not readily interpretable in terms of a model involving selective neutrality of all protein polymorphisms, but rather suggests the influence of selection acting either to stabilize frequencies at the geographically homogeneous loci and/or to produce interpopulation heterogeneity at other polymophic loci.

The identification of retinochrome in Loligo pealei. LINDA SPERLING AND RUTH HUBBARD.

Hara and Hara have described a photosensitive pigment in the inner segments of the retinal photoreceptors of the squid, *Todarodes,* which they named retinochrome (*Nature,* **206**: 1331, 1965). We have now identified retinochrome in *Loligo pealei* and confirmed their main findings (*cf. Nature,* **214**: 572, 1967; **219**: 450, 1968). Retinochrome has an all-*trans* retinaldehyde chromophore that is isomerized by light to 11-*cis,* the opposite of rhodopsin, whose 11-*cis* retinaldehyde chromophore is photoisomerized to all-*trans.* Retinochrome is a pH indicator with λ_{max} at 502 nm in acid and 380 nm in alkaline solutions (pH near 9). It resembles squid metarhodopsin (*cf.* Hubbard and St. George, *J. Gen. Physiol.,* **41**: 501, 1958). The 11-*cis* photoproduct of retinochrome is also pH sensitive, with λ_{max} near 475 and 370 nm and a pK near 6. Another unstable photoproduct has λ_{max} 440 nm, but its relationship to the 11-*cis* product is not clear.

Retinochrome is located in membranes that are continuous with rough endoplasmic reticulum and run down the length of the inner segment, perpendicular to the bulk of rhabdom membrane (Zonana, *Bull. Johns Hopkins Hosp.,* **109**: 185, 1961), and is screened from incoming light by rhodopsin and by black pigment granules in the rhabdom. We see no difference in the amount of retinochrome present in eyes that are light or dark adapted *in vivo.* However, retinochrome can be bleached in frozen and thawed eyes, in which the retinal elements have become disoriented. It is therefore very unlikely that the photosensitivity of retinochrome has physiological significance. The continuity of the inner segment membranes with endoplasmic reticulum suggests that retinochrome may be a membrane-bound precursor of rhodopsin.

Supported in part by a NSF grant to George Wald and by grants from the National Eye Institute to Ruth Hubbard and to George Wald.

An effect of acetylcholine on the central nervous system of the crab Carcinus maenas. A. L. SORENSON.

The presence of acetylcholine, its release, and the presence of its synthesizing and inactivating enzymes have already been demonstrated for the thoracic ganglion of *Carcinus maenas.* Other workers have also found that drugs such as atropine, curare, and eserine have marked effects when injected into Crustaceans. However, previous attempts to demonstrate a primary criterion of a chemical transmitter—an effect of acetylcholine on the central nervous system of Crustaceans—have led to equivocal results since the concentrations necessary to obtain a response were rather high (10^{-3}g/ml or about 5.5 mM). The present experiments show that when acetylcholine is applied to the thoracic ganglion of an exsanguinated crab *via* perfusion with crab saline through the sternal artery, leg movements can be produced in response to even smaller concentrations of this agent (10^{-6} to 10^{-4}g/ml) while topical application of acetylcholine at 10^{-3} g/ml is ineffective. Furthermore these responses to acetylcholine are potentiated by eserine.

Direct application of acetylcholine to the neuropile *via* electroosmotic injection from glass microelectrodes is also effective. This procedure produces potential changes which can be monitored by an adjacent glass microelectrode in the extracellular space. These changes in electric potential are probably due to current flow between areas of active and inactive membrane. These electrical responses are graded with respect to intensity of stimulus and furthermore, low frequency stimulation (1/sec) leads to a loss of the response. This latter effect may be due to desensitization since a few seconds without stimulation suffice for the return of the response.

These results show that acetylcholine, applied in moderate doses, has an effect on the central nervous system of the crab. The primary criterion for identification of acetylcholine as a synaptic transmitter in the crab central nervous system is thus fulfilled.

Presynaptic actions of Ca and Mg and postsynaptic actions of glutamate at a sensory synapse. A. B. STEINBACH AND M. V. L. BENNETT.

Ampullae of Lorenzini of skate are electroreceptors in which receptor cells form chemically transmitting ribbon synapses on sensory nerve terminals. Depolarization of presynaptic membrane evokes graded depolarizing postsynaptic potentials (PSP's) which initiate propagated spikes in afferent fibers. Presynaptic hyperpolarization can produce postsynaptic hyperpolarization, apparently by reducing tonic release of excitatory transmitter. The canal of single dissected ampullae was drawn into a suction electrode to electrically stimulate receptor cells. A second suction electrode monitored postsynaptic activity. Sensory synapses could thus be exposed to rapid solution changes. Saline contained 245 mM NaCl, 3 mM KCl, 350 mM urea, 5 mM glucose, 0.5 to 25 mM $CaCl_2$ and $MgCl_2$ and PO_4 or TRIS to pH 7.6. With Mg and Ca at 3 mM, PSP's were quantitatively like those in CSF. Magnesium at 5–10 mM increases threshold for evoking PSP's and decreases PSP amplitude. Calcium can partially cancel this effect. Magnesium at 10–25 mM blocks PSP's regardless of Ca. Calcium at 5–25 mM prolongs PSP duration without large changes in threshold or amplitude. Low divalent ion concentrations depress PSP amplitude although nerve excitability increases. L-glutamate at 10^{-6}–10^{-3} M initially (within seconds) enhances postsynaptic spike frequency and then rapidly depresses PSP's and eliminates all responses (reversibly with washing). L-aspartate, DL-homocysteic acid, and D-glutamate at 10^{-4}–10^{-2} M produce similar effects. Acetylcholine, dopamine, GABA, glycine, gallamine, d-tubocurarine and edrophonium produce no effects at 10^{-3} to 10^{-2} M. Effects of glutamate occur in low divalent ions confirming separate sites of action.

Spatial organization and adaptational changes of ON-OFF ganglion cells in Mustelus *retina.* WILLIAM K. STELL, PETER B. DETWILER, HENRY G. WAGNER AND MYRON L. WOLBARSHT.

Ganglion cell unit discharges, evoked by 500 msec flashes of white light, were recorded from the dogfish retina. Pieces of ventral eye cup were suffused with moist 100% oxygen at 20–22° C. Action potentials were recorded extracellularly with glass-coated Pt-Ir microelectrodes inserted through the vitreous body, amplified and displayed conventionally. Receptive fields were mapped by determining thresholds with spots of light 190 μm in diameter at different points along orthogonal axes, and the relation between threshold intensity and spot diameter (*i.e.,* the summation function) was determined for spots centered on the receptive field.

In most dark-adapted units, central spots gave ON-responses while surrounding annuli gave OFF-responses and inhibited the central ON-discharge. Some of these units were double-opponent, since under certain conditions they responded at OFF as well as ON in the center, and at ON as well as OFF in the surround. Usually the ON threshold for a small spot was constant across the receptive field center and rose sharply beyond a diameter of 1–2 mm. The OFF threshold, on the other hand, showed little change across the full 9.5 mm stimulus field. The central ON threshold decreased linearly with spot area up to a diameter of 2–3 mm, but showed no further change or increased with larger diameters. The OFF threshold, on the other hand, decreased linearly with area for spots of all sizes.

A steady diffuse background or flashed annulus (I.D. = 7.6 mm) strongly suppressed central ON sensitivity, and in some units reduced the diameter of the ON center as judged by both the receptive field map and the summation function. A steady background also usually enhanced OFF sensitivity.

Fine structure of the acrosomal region in spermatozoa of two echinoderms, Ctenodiscus *(starfish) and* Thyone *(holothurian).* R. G. SUMMERS, L. H. COLWIN, A. L. COLWIN AND R. TURNER.

The acrosomal region in both *Ctenodiscus* and *Thyone* is deeply embedded within the apical portion of the nucleus and is bounded anteriorly by the plasmalemma and posterolaterally by the nuclear envelope. Two components are present within the acrosomal region of both species: an acrosomal vesicle with a completely bounding membrane, and the periacrosomal material which surrounds the vesicle. The limiting membrane of the vesicle shows apical, lateral and basal differences with respect to associated materials; also, basally the membrane shows a slight depression or indentation. When sperm are fixed with osmic acid, the acrosomal vesicle membrane exhibits discontinuities. However, when sperm are fixed with glutaraldehyde, the acrosomal vesicle membrane is generally complete.

The contents of the acrosomal vesicle exhibit species differences in electron opacity after glutaraldehyde fixation. In *Ctenodiscus,* the contents are homogeneous except for a disc-shaped, lucent area at the base of the vesicle. In *Thyone,* an extensive meshwork of fibrous material resides within the base of the vesicle. The periacrosomal material in both species is homogeneous except for a dense, sub-acrosomal specialization which lies posterior to the vesicle. In *Ctenodiscus,* sub-acrosomal material is granular and in *Thyone* it is fibrillar.

In both forms the acrosomal vesicle has a completely continuous bounding membrane (as has *Asterias forbesi*—Longo and Anderson, personal communication) so placed that it could participate in the acrosomal reaction by fusing with the sperm plasma membrane. Thus, the reaction could follow the pattern found in *Saccoglossus* rather than the one suggested by Dan for the acrosomal reaction in echinoderms.

During the acrosomal reaction in *Thyone* the membrane of the acrosomal vesicle becomes continuous with the sperm plasmalemma; the vesicle releases its contents and everts to form a tubule. Periacrosomal material becomes the contents of the tubule which are predominantly fibrillar.

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Changes in ionic permeability during early development of the starfish, Asterias forbesi. JOSEPH T. TUPPER AND JOHN W. SAUNDERS, JR.

The membrane potential of the starfish embryo, as determined by intracellular microelectrodes, exhibits an increasing negativity during early cleavage. This change may arise due to alterations in passive ionic permeability, ionic distribution, electrogenic pumps or a combination of any or all these factors. However, application of the constant field theory to the observed ionic properties under a variety of conditions has led to the conclusion that the increased negativity results from a relative change in the embryonic permeability to K^+ ion.

Shortly after fertilization (resting potential approximately −50 mv) the change in membrane potential during a tenfold change in external K^+ is +35 mv. At the four-cell stage (resting potential approximately −70 mv) the change is +51 mv. The value for a K^+ electrode under these conditions is +56 mv. Therefore, the magnitude of the resting potential increasingly reflects the K^+ ion distribution. Plots of $e^{VF/RT}$ *versus* external K^+, in conjunction with the constant field equation, allow an estimate of internal K^+ and the ratio of Na^+ to K^+ permeability (P_{Na}/P_K). These plots predict an internal K^+ activity of approximately 200 mM at both the single cell and the four-cell stage. This has been independently confirmed by flame photometric analysis, assuming all the K^+ is involved in the electrochemical potential. Furthermore, this treatment of the data predicts a decrease in the P_{Na}/P_K ratio during early cleavage, resulting in a relative increase in K^+ permeability as related to Na^+ permeability. This in turn predicts the observed behavior of the membrane potential on the basis of passive ion permeability alone.

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Satellite DNA analysis in the spider crab Libinia emarginata. JACK C. VAUGHN.

Crab DNA was isolated by a chloroform-isoamyl alcohol procedure, treated with RNA-ase, α-amylase and pronase, sonically sheared and eluted from hydroxylapatite columns. Spectrophotometric thermal denaturation of this DNA in SSC/10 shows a biphasic profile. The dAT satellite DNA component is present in sperm, eggs, testes, muscle, hepatopancreas, hemocytes, gills and zoeae in virtually the same proportion (9%), with a T_m of 50.5° C. The T_m of main band DNA is 70.5° C. Normal probability plots reveal two main band DNA sub-families: an $(A + T)$-rich (11%) and a $(G + C)$-rich fraction (2%). Main band DNA has a 2σ value of 9.3° C. Hemocyte chromatin was isolated and separated into two fractions: condensed and disperse, whose purity and fibrillar morphology were demonstrated by electron microscopy. The protein/DNA ratio of each fraction is <2.3. Each chromatin melts giving a biphasic profile, with T_m's at about 69° and 83° C, showing that both main band DNA and dAT are complexed with protein (presumably histone) which protects them from melting and suggesting that dAT protein may be different from main band protein. DNA from these chromatins melts exactly like total tissue DNA, showing that dAT is equally distributed among condensed and disperse chromatin. Extraction with 2 M NaCl completely dissociates the dAT-protein and the main band DNA-protein complexes, showing that these bondings are ionic. Controlled partial melting of unsheared DNA in the presence of 12% HCHO specifically and irreversibly denatures dAT. Electron microscopy reveals denaturation loops, which presumably correspond to the dAT satellite sequences. These loops are about 0.5 μ long, and should each contain about 1500 nucleotide pairs with a molecular weight of 1×10^6 Daltons. The existence of larger (or smaller) loops can not be ruled out.

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Studies on the incorporation of sulfate into carrageenan in Chondrus. GEORGE WAGNER, MONICA LIK-SHING TSANG, JEROME A. SCHIFF AND F. LOEWUS.

The biosynthesis and biochemial characterization of carrageenan in *Chondrus crispus* is under investigation. $^{35}SO_4^{2-}$ is readily incorporated into carrageenan by the intact plant and the rate of the process is dependent on SO_4^{2-} concentration and time. Fractionation of the labeled polysaccharide into κ and λ fractions with KCl shows that both are labeled and that the sulfate is associated with high molecular weight polysaccharide polymers. Low molecular weight compounds are also formed from $^{35}SO_4^{2-}$ by *Chondrus* including small amounts of adenosine-5′-phosphosulfate (APS) and adenosine-3′-phosphate-5′-phosphosulfate (PAPS). Sulfation of carrageenan in cell-free extracts using synthetic $PAP^{35}S$ is also under investigation.

The authors gratefully acknowledge a Grant-in-Aid from Research Foundation of the State University of New York which was used to rent the counting equipment for this study.

Analysis of DNA synthesis during oocyte maturation and early cleavage in Asterias forbesii. PAUL M. WASSARMAN.

In the presence of 1-methyl adenine ripe females of *Asterias forbesii* are induced to shed oocytes which undergo germinal vesicle breakdown and meiotic maturation. We have used this system to investigate whether DNA synthesis (of either the repair or replicative type) occurs during the maturation period.

Starfish ovaries, washed thoroughly and incubated in the presence of ^{3}H-thymidine, were treated with 1-methyl adenine and, after 1 hr, the shed oocytes were harvested, washed and pelleted. The pellet was then lysed with SDS and in some cases the DNA was further purified by standard procedures. Equilibrium density centrifugation was carried-out on whole lysates after addition of solid CsCl, ethidium bromide and purified starfish sperm DNA as a marker. After centrifugation the contents of the tubes were fractionated onto paper discs and these were washed and counted in toluene scintillation fluid.

The radioactivity profiles of lysates of shed oocytes subjected to CsCl-ethidium bromide gradient centrifugation revealed incorporation of ^{3}H-thymidine into material which banded at the density of the nuclear DNA; no mitochondrial DNA synthesis was detected. Similar experiments carried-out in the presence of bromo-deoxyuridine indicated that the DNA synthesized during maturation had undergone a significant density shift. Thus far, however, these experiments have not completely resolved whether the synthesis represents DNA replication, repair, or both. On a sucrose gradient the newly synthesized DNA appears to have a much smaller molecular weight than purified starfish sperm DNA. The nature and function of the DNA synthesized concomitant with oocyte maturation remains to be established.

The CsCl-ethidium bromide gradient centrifugation method has also been used to determine whether mitochondrial DNA is replicated during early cleavage following fertilization of starfish eggs. The radioactivity profiles of such gradients clearly show that *no* mitochondrial DNA is synthesized until at least after the 128-cell stage.

This study was supported by NIH grant 5-TO1-HD00026-10 to the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory.

Sponge aggregation III: Isolation of a surface component required in addition to the aggregation factor. GEORGE WEINBAUM AND MAX M. BURGER.

Sponge factor may require its own achoring components on the cell surface which may have a high affinity for factor, acting thereby as receptors. The following effort supports such a notion.

The cell surface aggregation factor of *Microciona prolifera* was isolated by the chemical dissociation method of Humphreys and partially purified by differential centrifugation. Such chemically dissociated sponge cells became refractory to aggregation factor when treated with 0.08 M NaCl at 37° C for 20 to 30 min. with gentle agitation. Concomitantly the medium accumulated a component, probably released from the cell surface, which did not sediment at 105,000 *g* and which restored to the hypotonically shocked cells the ability to aggregate in the presence of aggregation factor. Additional evidence that the material released with 0.08 M NaCl had the properties of a surface receptor for factor comes from the observation that preincubation of factor with this material and then addition of control cells which had not been hypotonically shocked did prevent aggregation.

The release of the factor binding site was dependent on the hypotonic conditions, not occuring above 0.10 M NaCl. The release reached an optimum at 0.08 M NaCl. Hypertonic conditions (3 M KCl) did not induce release of the binding site.

A method for the specific isolation of factor binding site was developed utilizing affinity chromatography under conditions in which aggregation factor was covalently linked to Sepharose 4B. Such factor-coated beads may be useful in studying the initial steps in the aggregation process.

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Changes in the organization of tubulin during meiosis in the eggs of the surf clam, Spisula solidissima. RICHARD C. WEISENBERG.

Spisula eggs were homogenized at the desired times after activation in Kane's spindle isolation medium (1 M hexylene glycol at pH 6.3) and a total particulate fraction prepared by centrifugation at 50,000 *g* for 20 minutes. Pellets and supernatants were extracted in 0.1 M

KCl at pH 7, and the extracted tubulin determined by colchicine binding activity. The amount of particulate tubulin was about the same in unactivated eggs as in eggs at metaphase. The particulate tubulin was about 13% of the total tubulin in both unactivated and metaphase eggs. The amount of particulate tubulin was observed to decrease shortly after activation, reaching a minimum value at about 5 minutes, the time of nuclear membrane breakdown. The particulate tubulin concentration then rose, reaching a maximum at metaphase, and then decreased again at anaphase and formation of the first polar body. In the hexylene glycol homogenates of unactivated eggs a structure has been observed which has been shown to contain the interphase particulate tubulin (IPT). With phase contrast microscopy this structure appears as a granular sphere about 10 to 20 microns in diameter in which there is usually a clearer central region. The granular sphere is usually attached to a membranous structure which is probably part of the egg cortex. This structure is absent from homogenates after incubation of the eggs in 10^{-5} M colchicine or vinblastine, or after incubation at 0° C. These structures are also absent 5 minutes after activation of the eggs, when the nucleus is breaking down and the amount of particulate tubulin has reached a minimum. Since the IPT contains about the same amount of tubulin as the spindle, and breaks down immediately prior to spindle formation, it is likely that it is directly involved in the mechanism of spindle formation. The IPT is not likely to be composed of microtubules, and must instead be a new, polymorphic aggregate of tubulin.

Redistribution of beta-glucuronidase after fertilization of Arbacia punctulata *eggs.* GERALD WEISSMANN, STEVEN WECK, LAIRD P. CAGAN, WALTER TROLL, MILTON LEVY, AND AL GROSSMAN.

Beta-glucuronidase is a marker enzyme for lysosomes in most animal cells. To determine whether this enzyme was particle-bound in the mature oocytes of *Arbacia punctulata*, these were homogenized in 0.3 M sucrose, 0.3 M KCl, 3 mM EDTA, and 0.05 M phosphate buffer, pH 7.2. Granules sedimenting between 1000 and $15{,}000 \times g$ (M fraction) contained the bulk of beta-glucuronidase activity in "latent" form, which could be released upon treatment at acid pH and by Triton X-100. Discontinuous density gradient centrifugation in sucrose of the M fraction showed that beta-glucuronidase was concentrated in particles sedimenting with a density of 1.065 and 1.120. The latter, denser peak of activity coincided with the peak of TAMEsterase activity (see Grossman *et al*, this issue). Negative staining of gradient fractions with uranyl acetate, performed by Allen Bell, demonstrated that the 1.120 fraction contained large granules of 0.4 to 0.5 microns in diameter, which contained internal structures resembling those observed in cortical granules after sectioning. Smaller granules, 0.08 to 0.12 microns, were found in the 1.065 fractions. After fertilization by sperm, beta-glucuronidase activity underwent redistribution, with shift of the activity to less dense (1.040) and intermediate fractions (1.092). The latter peaks now showed coincidence of both beta-glucuronidase and TAMEsterase activity, with more of both enzymes appearing at 30 than at 5 minutes after fertilization. These fractions contained negatively stained organelles consistent with the simultaneous presence of large and small granules, some appearing merged. The data suggest that fertilization induces changes in the size and distribution of beta-glucuronidase-containing granules, perhaps because of merger and vesiculation of cortical granules and lysosomes.

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Differentiation without cleavage in an Ascidian egg: development of muscle acetylcholinesterase. J. R. WHITTAKER.

During normal development of the ascidian, *Ciona intestinalis*, a histochemically detectable acetylcholinesterase (method of Karnovsky and Roots) first occurs in larval muscle cells at 7½–8 hours of embryonic development (18° C). As reported previously by Durante, this is a tail muscle enzyme which does not develop elsewhere in the larva.

Cleavage is not necessary for the differentiation of this tissue-specific acetylcholinesterase. If fertilized eggs were prevented from undergoing cleavage by exposure to cytochalasin B (2 μg/ml), acetylcholinesterase differentiated in 5–10% of the eggs. Response was as high as 50–80% in occasional batches of eggs. Various cleavage stage embryos (2-, 4-, 8-cell, *etc.*)

which were similarly arrested with cytochalasin differentiated acetylcholinesterase in certain blastomeres. This differentiation followed the cell lineage pattern for larval muscle cells established by Conklin and Ortolani. Although the number of embryos responding was usually small at the blocked 1-, 2-, and 4-cell cleavage stages, essentially all of the embryos inhibited at the 8-cell stage developed acetylcholinesterase in 1 or 2 of the 2 muscle lineage blastomeres present at this stage. Embryos arrested at later stages always developed enzyme in some or all of their muscle lineage cells. Development of enzyme was slightly delayed in cleavage-arrested embryos: acetylcholinesterase in blocked 8-cell stages appeared at 9 hours development time.

Similar results were obtained when cleavage was inhibited with colchicine, Colcemid, or podophyllotoxin, but fewer embryos responded in the early cleavage stages.

Since nuclear division continues in cytochalasin-treated cells, the "muscle" blastomeres contain nuclei which belong in other cells. Obviously, these nuclei do not interfere with acetylcholinesterase expression. Acetylcholinesterase development may depend on nuclear differentiation but is apparently regulated by substances which are segregated into the appropriate blastomeres by cleavage.

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Escape reflex circuit in crayfish: interganglionic interneurons activated by the giant command neurons. JEFFREY J. WINE.

The four giant fibers in the crayfish nerve cord are the decision units of neuronal circuits which produce the rapid abdominal flexion often used to initiate escape swimming. Primary sensory fibers and interneurons presynaptic to the giants, as well as motoneurons postsynaptic to them, have previously been identified. This study continues the analysis of the circuit diagram by identifying interganglionic interneurons activated by the giant fibers.

Recordings were made from restricted portions of the desheathed, 2–3 connective of the isolated abdominal nerve cord of the crayfish. Giant fibers were directly stimulated with brief shocks and responding interneurons were characterized as fully as possible as to ganglionic origin; direction of spike propagation, extent of travel in cord, latency and number of impulses, and location in cross section of cord.

Preliminary results limited to units in the dorsal portion of the cord (Wiersma's areas 76 and 77) indicate at least eight interneurons. Units have been found to originate in all abdominal ganglia except the first, and all respond at short latency (1–15 msec, these values include conduction times). One unit usually responds with a short burst of impulses. This unit, which originates in the 2nd ganglion and appears to end in the 3rd, can also be activated by antidromic stimulation of the exclusively motor, ipsilateral 3rd root of the 2nd ganglion. Direct stimulation of this unit produces a small, slow depolarization in the lateral giant fiber which can be blocked by picrotoxin, hence this fiber is probably part of the circuit producing recurrent inhibition of the lateral giants.

Supported by a Fellowship in Neurophysiology from the Grass Foundation.

The neuromuscular basis of coxal feeding movements in Limulus. GORDON A. WYSE AND NANCY K. DWYER.

Movements of the leg coxal segments serve to masticate food. During feeding a coxa may trace a simple repeating arc of abduction and adduction around a dorsolateral pivot, or may take a more complex, oval path of elevation, abduction, depression, and adduction. This latter chewing movement with hysteresis aids in food transport into the esophagus. An additional pattern of reverse hysteresis removes food from the oral cavity. All coxal feeding movements, as well as promotion and remotion, are mediated by nine coxal muscles originating on the endosternite and on the dorsal prosomal shield. The actions of these muscles were determined by chronic electromyogram and tension recording in intact animals. In all feeding patterns, most muscles acted during adduction. Only muscle 26 consistently acted during abduction, but from anatomy and from direct stimulation, 26 is a promotor that does not abduct. Highly synchronous EMG activity occurred in four adductors (38a, 38p, 39, 40) during abduction in feeding. This synchronous activity did not correlate with tension development in the muscles and may represent peripheral inhibition. Hysteresis and reverse hysteresis were produced by muscle 29, and to a lesser degree, 27. These muscles pull radially to the main arc of adduction-abduction, and displace the pivot of that arc dorsolaterally. In feeding with hysteresis, activity recorded from these muscles tended to lag 90° in phase behind the main adductors; during feeding with reverse hysteresis it tended to phase-lead by 90°. These phase shifts would be sufficient to impart the observed circularity to the simple arc of abduction-adduction. The firing order and phase relationships of muscles in the same and in different coxae require several stable patterns of premotor drive, by mechanisms that remain to be studied.

This study was supported by grant NS 08869 from the USPHS.

Inhibition of dogfish lens protein synthesis by near UV photooxidized tryptophan.

TERESA YULO AND SEYMOUR ZIGMAN.

Former studies have shown that near UV light photooxidizes tryptophan to colored quinonoid products which bind to amino groups in many proteins, RNA and DNA. The present study was done to determine if the process of protein synthesis in dogfish (*Mustelus canis*) lenses would be inhibited by these photoproducts.

In each experiment, 2 fresh lenses of the eyes of medium fish were incubated at 22° C for 24 hrs under 3000 to 4000 μw/cm^2 of near (340 to 380 nm) UV light in elasmobranch Ringer's solutions containing 0.1% tryptophan and irradiated previously for 24 hr.

Controls were incubated in unaltered medium in the dark. Each flask contained 10 ml of medium plus 2 μc of C^{14}-amino acid mixture. After incubation, lenses were removed, washed, decapsulated, and homogenized in water. The total soluble and alpha, beta, and gamma crystallins were isolated as previously described, and specific activities were determined. Portions of and dialyzed and lyophilized total soluble protein were subjected to polyacrylamide disc-gel electrophoresis (Tris-glycine, pH 8.3), and stained with amido schwarz. Gels were sliced into 2 mm discs, the discs were dissolved in 30% H_2O_2, and the samples were counted. Similar acrylamide gel studies were done on the separated crystallins.

The proteins extracted from UV tryptophan treated lenses had specific activities only 20% of those of the dark controls. The radioactivity of all crystallin bands of the treated lenses were considerably lower than controls when gel discs were analyzed. Studies of isolated crystallins indicated a greater depression of alpha than of other crystallin syntheses, which were also markedly depressed. No difference in the permeability of lens capsules to alphaaminoisobutyric acid was found, indicating that loss of uptake by the lens was not involved. When a 0.001 M excess of ascorbic acid was added to media already containing photoproducts, a 5-fold increase in the incorporation of amino acids into the total soluble lens proteins over the UV-tryptophane treated lenses alone was observed.

It appears that the near UV-photoproduct of tryptophan is a potent inhibitor of lens protein synthesis *in vitro*, even more potent than actinomycin D. Although the mechanism of its action is not yet known, interference with DNA and/or RNA function and/or protein synthesizing enzymes is proposed.

Supported by The Rochester Eye and Human Parts Bank and the USPHS (Grant #EY-00459).

Continued from Cover Two

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3. ***A condensed title*** **or running head of no more than 35 letters and spaces should be included.**

Continued on Cover Three

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THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

HIGH FREQUENCY MUSCLES USED IN SOUND PRODUCTION BY A KATYDID. I. ORGANIZATION OF THE MOTOR SYSTEM[1]

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One of the most conspicuous insect songs heard in the Eastern United States is that of the katydid, *Neoconocephalus robustus*. *N. robustus,* like other tettigoniids, produces sound by rubbing a scraper on the edge of the right forewing across a set of teeth or file on the underside of the left forewing. The songs of most tettigoniids, and of gryllids which have a similar sound producing mechanism, consist of a series of readily discernible sound pulses, each pulse corresponding to a single stroke of the wings across one another. In a few tettigoniids, including *N. robustus,* the song is unusual in that it is a loud, continuous buzz. Oscillographic analysis of the song of *N. robustus* indicates that it too is composed of a series of discrete sound pulses, but the pulse frequency is sufficiently high, 150–200 per second, that the sound appears continuous to a human listener (Pierce, 1948; Alexander, 1956; Fig. 1 of this paper). This high pulse frequency indicates either that several sound pulses are produced for each wing cycle or that the frequency of wing movements is extraordinarily high. Evidence will be presented to show that the latter is the case; the wing frequency in a singing *N. robustus* is 150–200 per second with one sound pulse being produced per wing cycle.

Wing movements at frequencies exceeding 100 per second have been recorded during flight for a number of insects in several orders (Sotavalta, 1947). Such frequencies have hitherto generally been associated with asynchronous muscle (=myogenic muscle), a type of muscle peculiar to insects in which there is not a direct relation between the frequency of muscle contractions and the frequency of muscle action potentials. In the more usual synchronous muscle (=neurogenic muscle) each contraction is accompanied by one muscle action potential or a burst of action potentials. Generally the frequency of muscle action potentials in asyn-

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chronous muscle is considerably lower than that of the muscle contractions. Apparently the membrane activity of the muscle, indicated by the muscle action potentials, keeps the muscle in an active state during which it can oscillate at a frequency determined principally by the nature of the load (Boettiger, 1960; Pringle, 1967).

Asynchronous muscle has not been found in Orthoptera, the insect order which includes the tettigoniids. It was therefore of considerable interest to determine if the rapidly-contracting muscles used by *N. robustus* to move its wings during singing were synchronous or asynchronous. If the muscles were asynchronous, it would mean that this mechanism of muscle control is more widespread among insects than has been thought. If the muscles proved to be synchronous, their repetition frequency during singing would make them among the fastest synchronous muscles in the animal kingdom.

Materials and Methods

The animals used were adult male specimens of *Neoconocephalus robustus* collected from salt marshes in Falmouth, Massachusetts. The captured animals were fed lettuce and kept in cages outside the laboratory or near a window so they experienced approximately normal diurnal fluctuations in light intensity.

Muscle action potentials were recorded between electrodes implanted in the thoracic muscles and a bare silver wire in the abdomen. The muscle electrodes were 50 μ silver wires, insulated except at the tip. They were inserted through holes in the exoskeleton and sealed in place with dental wax. All electrodes were soldered to long leads of copper wire, 80 μ in diameter. These were light enough that they did not seriously hinder the movements of the animal but strong enough that they usually were not broken by the animal's movements.

Electrode implantation was done under CO_2 anesthetization. After the electrodes were in place, the animals were kept in inverted funnels, 15 cm in diameter, with the recording leads emerging from the spout of the funnel. The animals usually did not sing in the evening following electrode implantation, but they generally did on subsequent evenings. The muscle action potentials were amplified with conventional capacitor-coupled amplifiers and recorded on magnetic tape for later analysis and photography. The position of the recording electrodes was verified by postmortem dissection. In later experiments an electrode marking technique was used to avoid possible misinterpretations caused by electrode movement during the dissection. After an animal had sung and action potentials had been recorded, the animal was anesthetized with CO_2 and current was passed between each of the recording electrodes and the indifferent electrode, the indifferent electrode being the cathode. The animal was then fixed in a solution made of equal parts of 10% formaldehyde and commercial photographic developer (Kodak D19). The developer reduced the silver deposited from the electrodes by the current and left a small black spot to mark the position of the tip of each electrode. A current of 100 μA for 10 seconds produced spots easily seen with a dissecting microscope.

The temporal relations between muscle action potentials and wing movements were determined with a strobe light arranged so that it was triggered after a variable delay by the action potentials recorded from one muscle. By adjusting the delay while watching the animal, the strobe light flash could be made to occur when the wings reached the maximally open or maximally closed positions. An electrical pulse coincident with the light flash was recorded on the magnetic tape along with the muscle action potentials, thus marking the wing position.

Results

The frequency of sound pulses and wing movements

The song of *N. robustus* typically consists of a series of sound pulses at 150–200 per second. Each pulse is an envelope of sound, the frequency within the envelope being approximately 7 KHz (Fig. 1; Pierce, 1948). It is not known whether the 7 KHz frequency represents a resonant frequency of some part of the sound producing mechanism or the rate at which individual teeth on the file of the left forewing are struck by the scraper on the right wing (for discussion see Dumortier, 1964).

In some tettigoniids two sound pulses are produced for each wing cycle; one when the wings cross one another in closing and the other on the opening stroke (Dumortier, 1964). Alexander (1956) has suggested that the sound pulses of *N. robustus* may be paired with two sound pulses being produced for each wing cycle. If this were the case, the frequency of wing movements should be half that of the sound pulses, that is, 75–100 per second. We examined singing *N. robustus* with a stroboscope and found that in 48 bursts of singing from 10 animals the average wing frequency was 177 cycles per second (range = 145–200 cycles per second, 20–23° C). The correspondence between wing frequency and that of sound pulses indicates that generally one sound pulse is produced on each cycle of wing movement.

Most of the songs which we recorded were from insects in the inverted funnels used to hold animals during measurement of muscle action potentials. These songs were often more complex than those of Figure 1 with two or more sound pulses for each wing cycle. This may be a normal feature of the song, but could also be a result of echoes in the small chamber holding the animals. In some recorded songs occasional sound pulses are reduced or missing (Fig. 2). This suggests that sometimes the wings may move by one another without the scraper encountering the file and so without a sound pulse being produced.

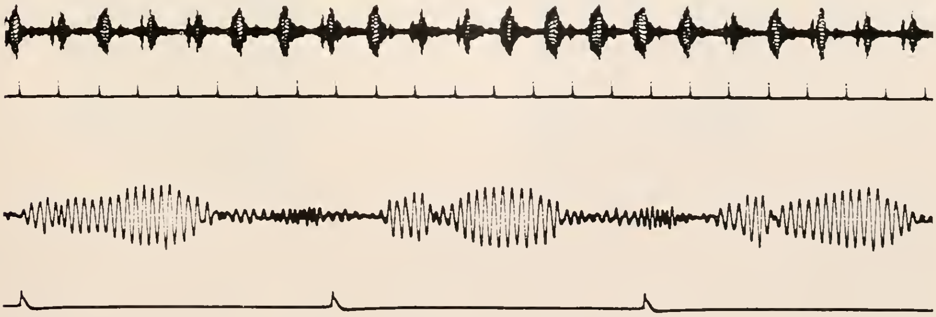

FIGURE 1. Sound pulses produced by a stridulating *N. robustus*. The time marks in this and following figures are 5 msec apart.

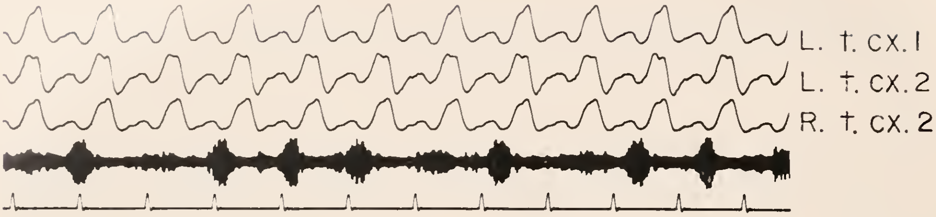

FIGURE 2. Sound pulses and muscle action potentials from three synergistic muscles. In this and other records showing action potentials, positive is up. Note the sound pulses are reduced or absent on some action potential cycles.

The frequency of muscle action potentials

Muscle action potentials recorded with implanted electrodes always occurred in essentially a one-to-one relation to sound pulses and wing movements (Figs. 2, 5, 6). Thus despite the high frequency of the wing movements the muscles used to move the wings are synchronous muscles.

Because of the high repetition rate the muscle action potentials recorded during singing appear as parts of a continuous wave rather than as distinct spikes. The electrical record is also complicated by cross talk from muscles adjacent to that from which the recording is made. Wing musculature makes up most of the mesothoracic volume and large portions of this musculature fire synchronously during singing. Some electrical pickup between muscles is probably unavoidable given that there is simultaneous activity of large blocks of muscle in a relatively small space. Distinct spikes, recorded in only one of several channels, are seen during the warm-up period preceding singing (Heath and Josephson, 1970) and sometimes at the onset or cessation of singing (see Figs. 5, 11, 12). These presumably represent activity in a single muscle with possible contributions from synchronously firing neighbors.

The extracellular action potentials are up to 10 mv in amplitude. The recorded potentials are principally positive when the electrode tip is firmly within the muscle; the potentials from electrodes lying on the surface of a muscle or between two muscles frequently have large negative components. The action potentials often have notches on the rising phase or multiple peaks. This might be due to electrical pickup from neighboring muscles or, more likely, to activity recorded from two or more functional units active nearly simultaneously within single muscles.

The possibility that the recorded potentials are movement artifacts cannot be rigorously ruled out but similar techniques applied elsewhere with insect muscles have been shown to be adequate for recording electrical activity of muscle (*e.g.*, Wilson, 1961; Bentley and Kutsch, 1966). The regularity and repeatability of the wave shape throughout bursts of singing and in successive bursts make it very unlikely that the recorded electrical events are movement artifacts rather than muscle action potentials. The cycle-to-cycle repeatability of the electrical record also indicates that the singing muscles are equally active on each cycle and rules out the possibility that the high frequency is due to an alternation of muscle blocks such that different groups of muscle elements are active on successive cycles.

The functional organization of the singing musculature

The muscles used in sound production, those which move the forewings, are shown in Figure 3. The arrangement of wing muscles in the mesothorax of *N. robustus* is similar to that described by Tiegs (1955) for the tettigoniid *Acridopeza reticulata* with the following exceptions: (1) in *Neoconocephalus* there is but a single basalar and single subalar muscle on each side, in *Acridopeza* there are three basalars and two subalars on each side; (2) in *Acridopeza* the tergotrochanteral muscle is of moderate size while in *Neoconocephalus* it is very small and probably does not play a significant role in sound production.

The pattern of electrical activity from the forewing muscles during stridulation is quite simple. All indirect flight muscles (the tergocoxals, tergosternal, pleuro-

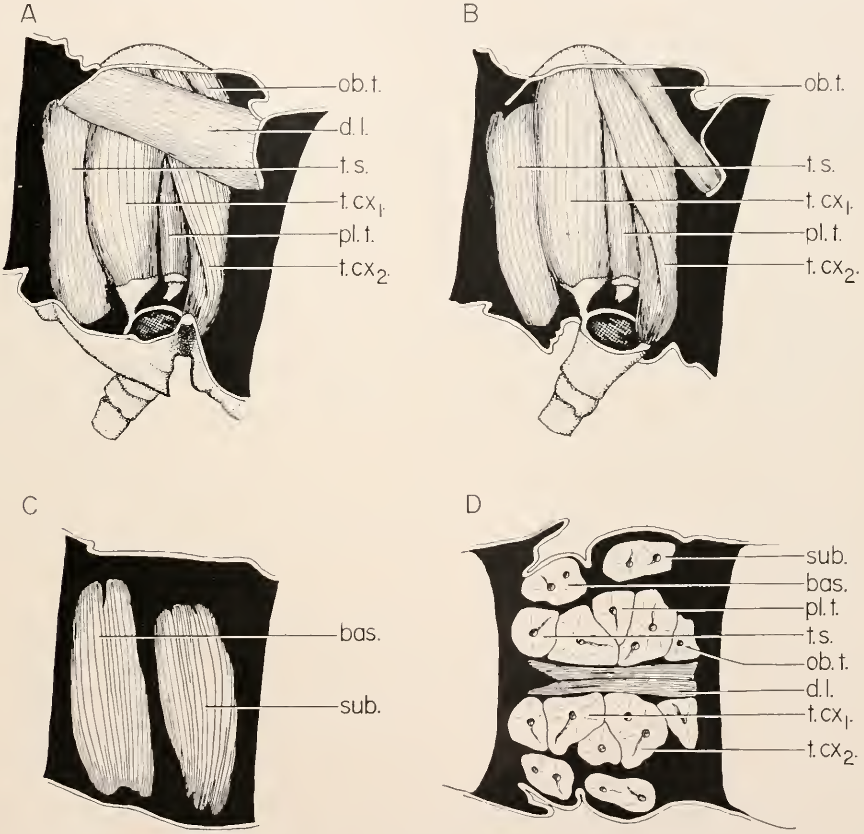

FIGURE 3. The musculature of the mesothorax. A is a median view of the muscles of the right side; anterior is to the left. In B and C progressively more of the medial musculature has been removed to expose lateral muscles. D is a horizontal section through the dorsal mesothorax. The tergotrochanteral muscle is not shown. The two thin branches of this muscle are lateral to the intersection of the first tergocoxal and the pleurotergal. The abbreviations used are: ob.t., oblique tergal; d.l., dorsal longitudinal; t.s., tergosternal; t. cx$_1$, first tergocoxal; pl.t., pleurotergal; t.cx$_2$, second tergocoxal; bas., basalar; sub., subalar.

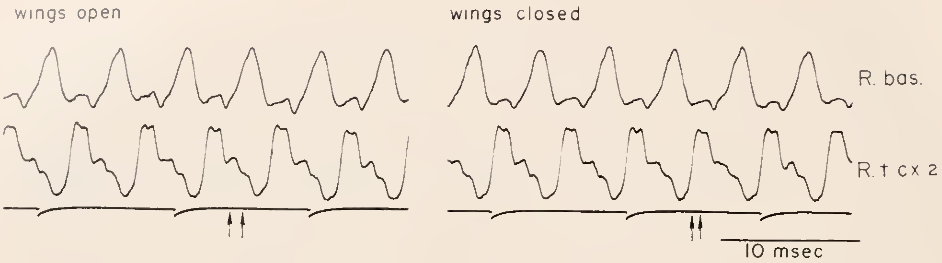

FIGURE 4. Strobe light determinations of wing position during singing. The strobe light was triggered by the muscle action potentials recorded in the upper channel after a delay which was varied so the light flash occurred when the wings were fully opened (left set of records) or fully closed (right set). The lower channel marks the time of the light flash. In these records the strobe light was triggered on every other wing cycle. The arrows mark the range for five separate determinations of wing position with this animal.

tergal, oblique tergal and dorsal longitudinal muscles) fire essentially synchronously. The direct flight muscles (the basalar and subalar muscles) also fire synchronously and in approximate antiphase to the indirect muscles. Stroboscopic determination of wing position during singing indicates that the direct flight muscles begin to fire when the wings are approximately fully opened and the peak of the muscle action potential occurs in the middle of the closing stroke (Fig. 4). Similarly action potentials from the indirect flight muscles begin when the wings are fully closed and the peak occurs during the opening stroke of the wings. Since the next movement following the potential peak from the direct flight muscles is wing opening, the direct flight muscles are presumably wing openers. By the same argument the indirect flight muscles are wing closers. Confirmation of this is given by activity patterns recorded at the cessation of singing. When singing stops, either spontaneously or in response to mechanical disturbance, the wings stop in the closed position. The indirect flight muscles are the last to fire when singing stops (Fig. 5); these must, therefore, be wing closers. When singing resumes, the first wing movement is opening and the first electrical activity appears in the direct flight muscles. These then are wing openers.

In the animal of Figure 4 the latency between the muscle action potential peak and the wing movement initiated by the action potential is approximately 2 msec. In isolated locust flight muscle the latency between the peak of the muscle action potential and the onset of contraction is about 1 msec. and is relatively independent of temperature (Neville and Weis-Fogh, 1963). The longer delay between the action potential and the onset of the initiated movement in *N. robustus* presumably represents time taken for the tension to rise in newly activated muscle until its effect is greater than that of the antagonistic muscles in which tension must be simultaneously falling. Sound pulses generally end about the time of the action potential peak in closer muscles, that is, about the time of maximum wing closure. This indicates that the sound pulses are produced on the closing stroke of the wing cycle.

The functional organization of the stridulation muscles in *N. robustus* is generally similar to that described for crickets (Bentley and Kutsch, 1966; Kutsch,

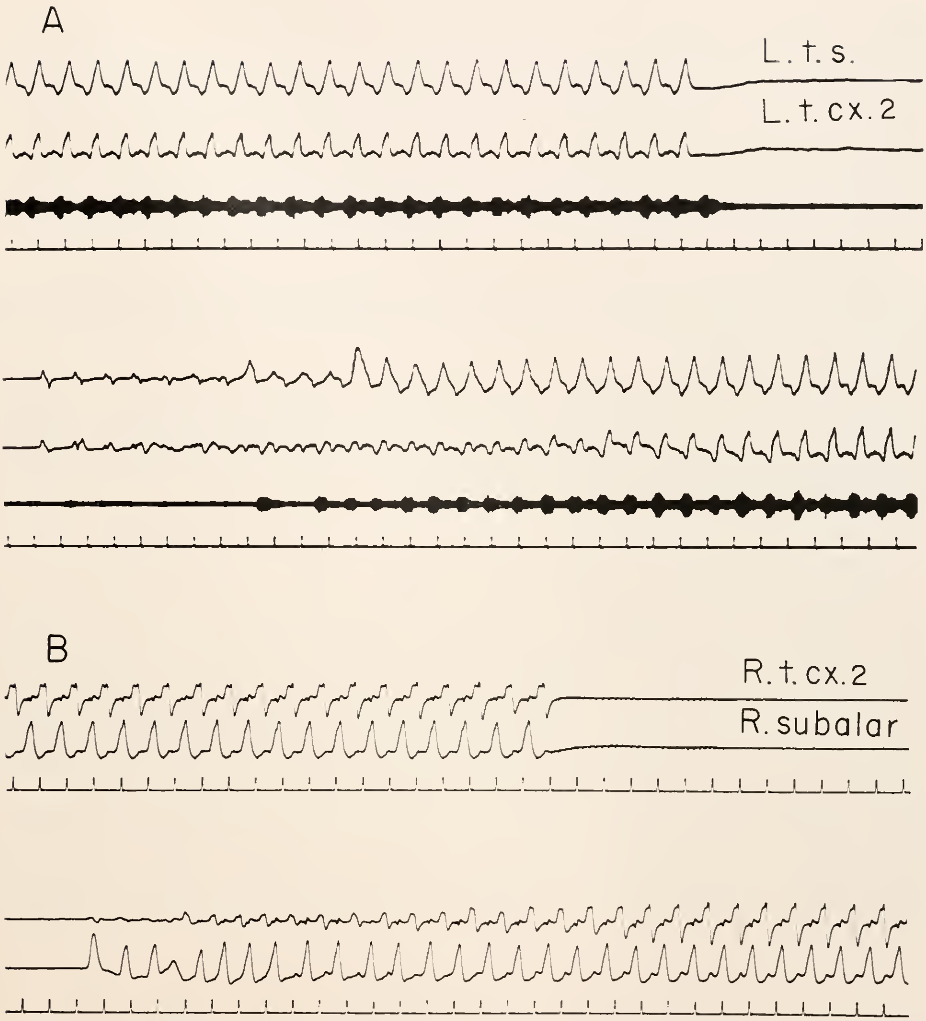

FIGURE 5. Singing pauses induced by tapping on the animal's container. In A and B, the upper sets of records are the cessation and the lower sets the resumption of singing. The singing pause in each case was less than one second.

1969) with direct flight muscles being wing openers, indirect flight muscles principally wing closers, and the sound pulse being produced on the closing stroke of the wings. It is surprising that in *N. robustus* the dorsal-longitudinal muscle is a synergist to the dorsoventral indirect flight muscles, but this is quite clear from the muscle recordings (Fig. 6). The wing movements during stridulation in crickets and tettigoniids are obviously related to those during flight (Bentley and Kutsch, 1966; Huber, 1962), and in insect flight the longitudinal and dorsoventral indirect flight muscles are generally antagonists (Pringle, 1957). There is a precedent for the arrangement found in *N. robustus*. In crickets the dorsal-longi-

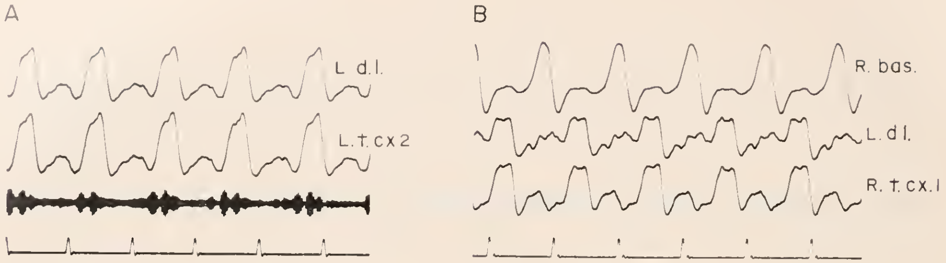

FIGURE 6. Action potentials from the dorsal longitudinal muscle during singing. Note that the dorsal longitudinal clearly fired synchronously with the tergocoxal muscles and is out of phase to the basalar.

tudinal muscle has two components, one of which contracts with the direct flight muscles and the other, as is the case for the single dorsal-longitudinal muscle of *Neoconocephalus,* contracts with the dorsal-ventral indirect muscles during stridulation (Bentley and Kutsch, 1966; Kutsch, 1969).

Activity patterns during singing

At the onset of singing muscle action potential patterns can be somewhat erratic. Once singing is fully established, the activity pattern is usually extremely regular. If an oscilloscope sweep is triggered by action potentials from one channel, with most animals successive sweeps fall directly on top of one another, indicating that the action potential frequency is regular and that the shape of the spikes constant from cycle to cycle (Fig. 7A). An electronic counter was used to measure the spike frequency in records collected from 32 animals. Ten intervals, each one second long, were measured for each animal, the intervals being separated by one second periods during which spikes were not counted. The average spike-frequency in these animals was 186.8 per second (s.d. = 13.3 per second, range = 157.6–212.6 per second). The coefficient of variation was computed for each of the animals as a measure of intra-animal variability. The average coefficient of variation was

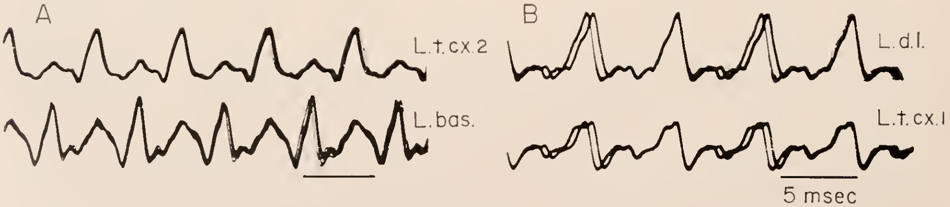

FIGURE 7. Regularity (A) and alternation of cycle lengths (B) during singing. In each case the oscilloscope was triggered by action potentials in the upper channel and the camera shutter left open long enough to superimpose 8–10 successive sweeps. The usual result when this is done is that in A, nearly exact cycle to cycle repeatability. B is from part of a record in which long intervals alternated with short intervals. In this record the oscilloscope sweep was arranged so that one sweep began with a long interval and the next with a short interval. Note that the time taken for any two consecutive cycles was nearly constant as shown by the nearly exact superposition of the second and fourth complete action potentials.

only 0.54% (s.d. = 0.30%). It should be pointed out that this is an underestimate of the regularity. The frequency was determined by counting the number of events in a fixed time interval. This method introduces some variability, for even if the frequency were perfectly constant the number of events counted could vary by one count depending on whether the interval began just before or just after an event. Further, some of the records were from near the onset of singing, a period when the frequency is not stationary but rising slowly (*e.g.*, Fig. 1 of Heath and Josephson, 1970) so the variability here would be greater than that which would be measured when a steady state is reached.

Although the overall frequency is extremely constant, there is sometimes interesting micro-structure in the patterns. In about one-third of the available records the interval between successive action potentials was not constant but alternated between long and short values during some part of the singing period. Alternation, when it occurs, is most pronounced early in the singing period and gradually disappears as singing progresses. Activity in synergistic muscles was always coincident; if there was alternation in the interspike intervals it was the same in each recording channel (Fig. 7B). Alternation in interval length was seen five times in recordings which were made simultaneously from antagonistic muscles. In one of these alternation was seen only in the opener muscle and the closer muscle intervals remained constant. The converse was seen in one animal, with alternation

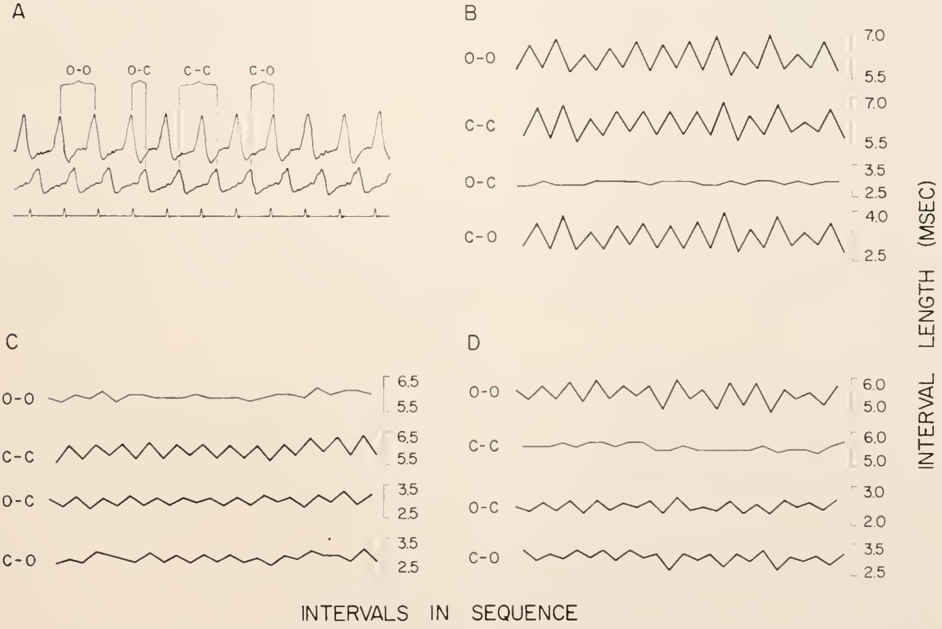

Figure 8. Patterns of activity in animals with alternating long and short cycles. The intervals measured are illustrated in A, which shows muscle action potentials from an opener (left subalar, upper channel) and a closer (right second tergocoxal, lower channel). The most extreme alternation encountered is that shown in B. Note that the opener-closer intervals are nearly constant in B as are the opener-opener and closer-closer intervals in C and D, respectively.

being confined to the closer muscle. In three animals alternation occurred in both opener and closer muscles. In these records the intervals between closer action potentials and those between opener action potentials alternated between long and short values but the intervals between an opener spike and the following closer spike were constant. Examples of these patterns are seen in Figure 8 and their implications as to the central organization of elements producing the activity are considered in the discussion.

One animal produced an unusual activity pattern in which rhythmic electrical potentials, obviously in the singing pattern, occurred in only one of two antagonistic forewing muscles being monitored (Fig. 12C). This "singing" occurred in short bursts following apparently normal warm-up activity. No sound was produced. The potentials from the closer muscle were typical of singing. The opener muscle produced no large spikes although these were present during warm-up. Low level activity in the opener channel, however, suggests that other opener muscles were firing in their normal sequence. This indicates that in the command chain there are points of lability at the level of individual muscles or motorneurones which can result in a muscle failing to participate in the usual activity pattern.

Warm-up and the transition to song

Singing is preceded by a warm-up period during which the forewing muscles are active. During warm-up the wings are held in the resting rather than singing position and normally antagonistic muscles fire synchronously so no wing movement or sound is produced (Fig. 9; Heath and Josephson, 1970). The thoracic temperature rises at about 1.5° C/min during warm-up and at the onset of singing the thoracic temperature is about 33.5° C.

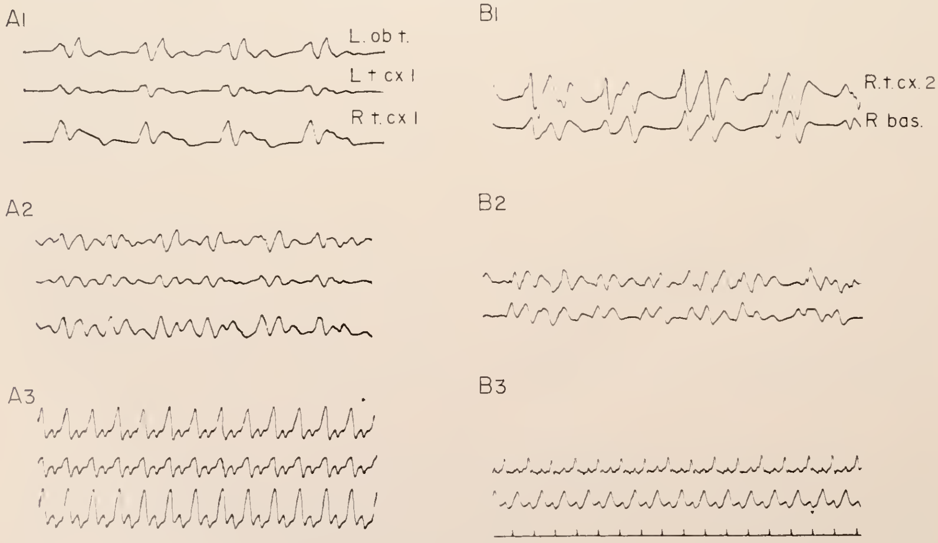

Figure 9. Warm-up and singing in synergistic muscles (A) and antagonists (B). The upper sets of records are from early warm-up, the middle sets from late warm-up and the lower sets show fully-established singing. The 5 msec time signal in (B3) applies to all records.

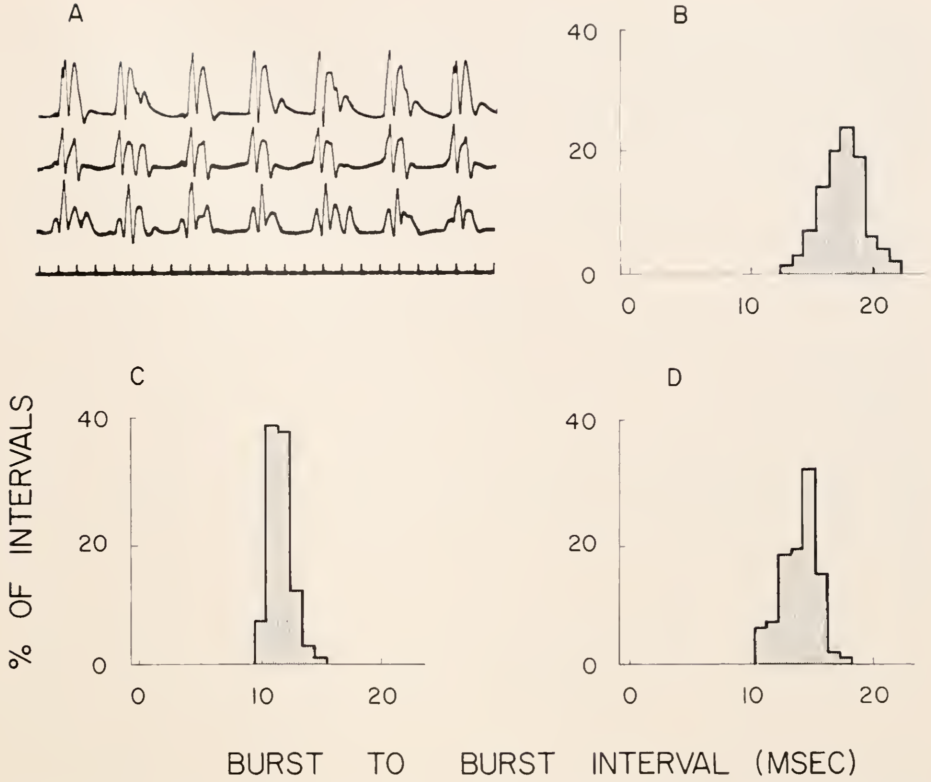

FIGURE 10. Inter-burst interval distribution during early warm-up. Each histogram includes 100 successive intervals measured from the onset of one burst to the onset of the following burst. The records chosen for analysis were ones in which the bursts appeared rather regular in a preliminary inspection. A portion of the original record from which D was obtained is shown in A. The muscles here are the right tergosternal (upper), the right first tergocoxal (middle) and the left dorsal longitudinal (lower channel).

Initially warm-up is intermittent with periods of muscle activity several seconds to a few minutes long separated by silent periods. Later the activity is not interrupted by significant pauses. Through most of warm-up the muscle action potentials occur in short bursts of two to four individual potentials, seen clearly in some records but tending to fuse in others. The interval between the onset of successive bursts is 10–30 msec. The bursts can occur quite regularly (Fig. 10). The bursts might be due to either individual motor units in each muscle firing in slight asynchrony or to multiple firing by the same population of units. The latter seems more likely. The intervals between successive peaks of a burst can be quite regular, suggesting repetitive firing. Further, simultaneous recordings from different muscles usually show exactly the same number and spacing of pulses in equivalent bursts, even when the recordings are made from muscles which are antagonists in singing. This similarity would require that each muscle has the same number of motor units which are activated in the same pattern if the separate peaks are due to multiple units rather than repetitive firing. As warm-up proceeds, the burst frequency may increase somewhat, but the frequency change is less than might be expected from the rising thoracic temperature. In one animal from which electrical recordings were made the thoracic temperature was also measured with an implanted thermistor (see Heath and Josephson, 1970, for details). In this animal the burst frequency was 56 per second early in warm-up when the thoracic temperature was 26° C and had increased to only 68 per second when the thorax had warmed to 31° C. Muscle contraction frequency during warm-up by the moth, *Hyalaphora cecropia,* is similarly only slightly dependent on thoracic temperature (Hanegan and Heath, 1970). Late in warm-up the muscle activity usually loses its bursty character and the recorded potentials appear as a continuous ripple. Regular bursts are interspersed with periods of continuous activity at the transition between bursting and continuous activity. It seems as though the transition from bursting to continuous activity results from an increase in the number of pulses in each burst so that activity continues through the interburst period. One animal produced only short bursts of potentials throughout warm-up; in all others the activity became continuous for some time prior to the onset of singing. Muscle potentials stop abruptly for a brief period just preceding the onset of song (Fig.

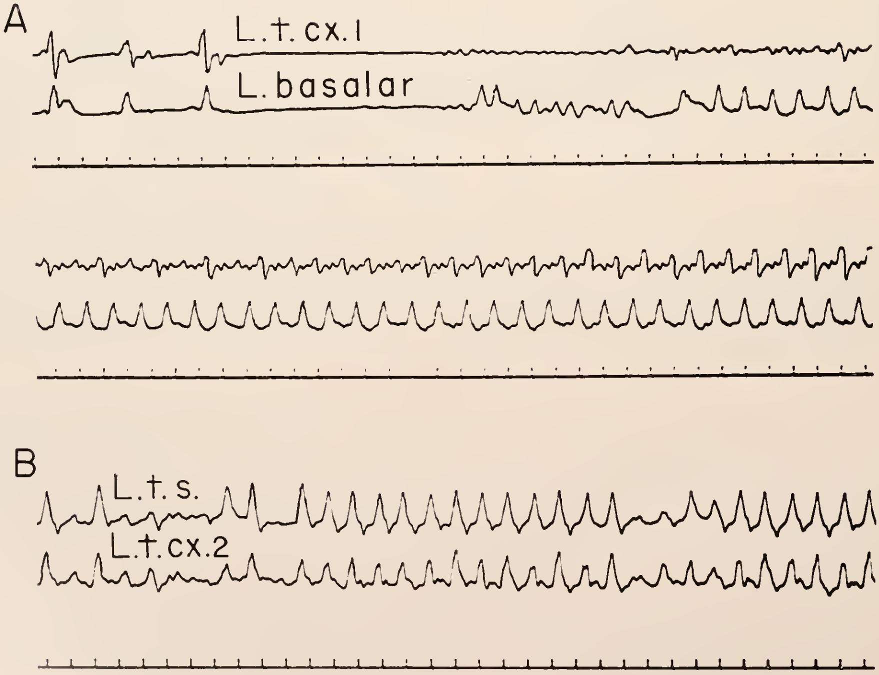

FIGURE 11. The transition from warm-up to song. The lower set of records in A is a continuation of the upper set (part of one cycle is missing). Note the pause in A between the end of warm-up and the onset of singing. Activity recorded near the onset of singing from another animal is shown in B. Here there was missing of occasional cycles. Another example of missing during early singing is seen in Figure 2 of Heath and Josephson (1970).

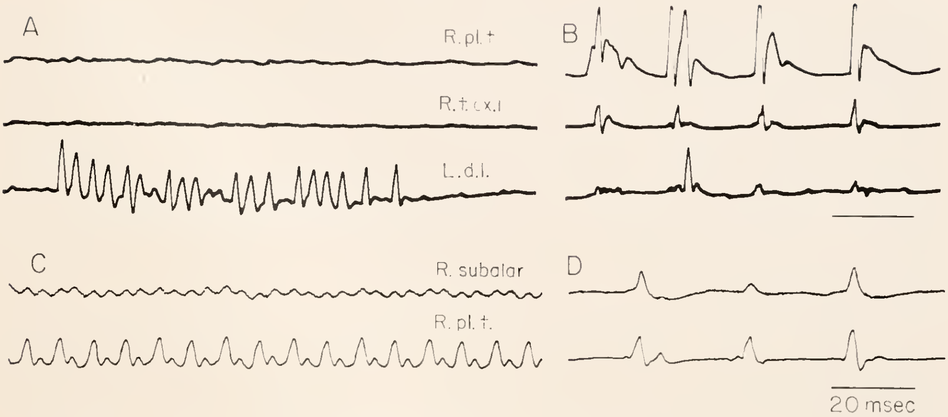

Figure 12. Some unusual activity patterns. A is from early warm-up and shows a short burst of spikes restricted to one channel. B was recorded slightly later from the same preparation. It is included because it shows that all three channels were operative and is another example of activity not synchronous in all channels during warm-up. The activity in the lower channel of C was clearly the singing pattern but in the upper channel only low level activity was recorded, indicating that this muscle was not firing. This pattern, which occurred without sound, followed apparently normal warm-up several times in this animal. D is from the same animal shortly later and again shows that both channels were operative.

11A). The duration of the pre-singing pause averaged 64 msec (range = 25–110 msec) in five recorded transitions from warm-up to song.

A striking feature of warm-up is the usual synchrony in activity recorded from different muscles. In all animals occasional bursts may have more or fewer peaks in one channel than in others but, as indicated above, the synchrony usually extends even to the number and spacing of pulses in bursts. It must be emphasized that the deviations from coincident activity now to be considered are exceptional. In two animals, in both of which activity was monitored simultaneously from three muscles, one muscle consistently produced single spikes each time the other two produced bursts. In some animals one muscle produced single spikes and short bursts of spikes, occasionally at frequencies exceeding those of singing, which were not seen in other channels (Fig. 12). While probably not of great significance for singing behavior, these instances of activity isolated to one channel indicate that there is not inescapable coupling between motor neurons during warm-up. Further, recorded events restricted to one channel show that there is often little inter-channel coupling due to electrical fields created within the thorax.

The first rhythmic potentials characteristic of singing are seen in opener muscles but not all opener muscles begin simultaneously; one may fire several times before another begins. Asynchrony in the onset of the singing pattern is even more pronounced in the closer muscles and most forewing muscles may be firing regularly and sound production have begun before an individual closer muscle joins in (Fig. 5A). There may be facilitation of pulse amplitude over a number of cycles after the onset of the singing pattern. This facilitation is more obvious in closer than in opener muscles. In a related tettigoniid, muscle action potentials from isolated forewing muscles show no facilitation (Josephson, in preparation), suggesting that the increase in action potential amplitude recorded at the onset of singing may result from recruitment of motor units within the muscle, possibly with cross-talk contributions from neighboring muscles. Near the onset of singing, activity recorded from a muscle may occasionally miss a cycle or a number of consecutive cycles, up to 10 (Fig. 11B). This skipping of cycles seems to involve the whole set of synergistic muscles. At the expected time of firing there are no small deflections in the records of the sort which result from cross talk from nearby active muscles. When two synergists are monitored, both skip cycles together while antagonists continue regular firing. This skipping must, therefore, result from lability in some part of the command chain common to the motor neurons forming the synergistic group.

As indicated above, singing usually stops abruptly with the closer muscles being the last to fire. Occasionally the closer muscles produce an extra spike or two at the end of singing and in one animal a single closer muscle continued to fire irregularly after the singing activity had stopped in other channels.

Discussion

Repetition frequencies of synchronous muscles

Despite the high wing frequency during singing there can be no doubt that the wing muscles of *N. robustus* are synchronous. There is clearly a one-to-one relation between muscle action potentials and wing strokes. Further, the muscle ultrastructure is entirely consistent with that predicted for a fast, synchronous muscle (Elder, 1971). The sarcoplasmic reticulum is extremely well developed and the myofibrils are thin, minimizing diffusion distances from the center of a myofibril to the nearest sarcoplasmic reticulum. Finding a muscle action potential for each contraction does not necessarily mean that there is an antecedent motorneurone impulse for each contraction. In bumble bee flight muscle a single motorneurone impulse can evoke a junctional potential which produces several muscle action potentials (Ikeda and Boettiger, 1965). The strict synchrony between synergistic muscles in *N. robustus,* however, cannot be accounted for on the basis of repetitive firing in independent units; some central synchronizing mechanism is necessary. Thus in *N. robustus* there is almost certainly a one-to-one relation between contractions and impulses in participating motorneurones; the muscles are, as is the usual case, both synchronous and neurogenic.

The wing frequency of *N. robustus* during singing is 145–212 per second. In contrast, the highest wing frequency during flight reported for animals presumably using synchronous muscles is that of some moths (Aegeridae) whose wing frequency reaches or slightly exceeds 100 per second (Sotavalta, 1947). It is interesting that with the exception of these moths and the asynchronous muscles of insects, all muscles which have been found to have repetition frequencies exceeding 100 per second are involved in sound production. The synchronous tymbal muscles of cicadas (Hagiwara, 1956; Aidley, 1969) and the sound producing muscles of the toadfish, *Opsanus tau,* (Skogland, 1961), the squirrel fish, *Holocentrus rufus,* (Gainer, Kusano and Mathewson, 1965; Winn and Marshall, 1963), and the lobster, *Homarus americanus,* (Fish, 1966; Mendelson, 1969) all contract at frequencies near to or greater than 100 per second during sound production. The sound producing muscles of midshipman, *Porichthys notatus,* (Cohen and Winn, 1967) and the cricothyroid muscle of the bats *Epitesicus fuscus* and *Myotis lucifigus* (Revel, 1962; Griffin, 1958) can reach or exceed contraction frequencies of 200 per second. The performance of sound-producing muscles in fish and in the lobster is the more remarkable when it is considered that the high frequencies are achieved at temperatures considerably lower than is the case with bats or with *N. robustus* in which the thoracic temperature is about 35° C during singing (Heath and Josephson, 1970). Indeed the sound producing muscle of the lobster has the most extensively developed sarcoplasmic reticulum yet described; here the sarcoplasmic reticulum comprises approximately 75% of the muscle volume (Rosenbluth, 1969). However, it is an insect which seems to offer the highest repetition frequency yet found for synchronous muscle. The bush cricket, *Orocharis gryllodes*, stridulates with its forewings. The wing frequency during stridulation is a function of ambient temperature and reaches 280 per second at 35° C (Walker, 1969). Although it has not been directly demonstrated it seems likely that the singing muscle is synchronous because (1) asynchronous muscle has not been found in the large number of orthopteran insects which have been examined, and (2) of our results in this paper indicating that insect synchronous muscle can exceed repetition frequencies of 200 per second.

The endogenous origin of the motor patterns

The forewing muscles in the mesothorax of *N. robustus* are involved in several activity patterns which differ in the frequency of muscle activation, the temporal organization of the muscle action potentials (single impulses at regular frequency or impulse bursts) and the phase relations between different muscles. Two activity patterns, those of warm-up and singing, are described here. The same muscles involved in these are also used for flight, during which the muscles are driven at a much lower frequency than during singing and in different phase relations than during warm-up. The tergocoxal muscles, from their morphology, may also be used during walking; the first tergocoxals being coxal protractors and the second tergocoxals being coxal retractors (Fig. 3). If these muscles are used during walking they may simultaneously participate in two activity patterns for *N. robustus* frequently walks about during warm-up and it can shift its position while singing without there being a noticeable pause in the song.

Three basic mechanisms have been proposed for the origin of rhythmic motor patterns in animals (for reviews see Wilson, 1964; Hoyle, 1964): (1) Reflex chains in which the consequences of the motor activity in one part of the cycle initiate sensory inflow which triggers the next part of the cycle. (2) Stored patterns of expected sensory input (sensory tapes (Hoyle, 1964) or sensory templates (Wilson, 1968a)) to which actual sensory input is compared, any discrepancies initiating a compensating motor output. (3) Endogenous generators which produce repetitive series of appropriate motor commands without the necessity of sensory inputs as timing cues. In the last, sensory information does not have a direct role in the sequencing although it may be necessary to initiate a pattern and it may modulate activity in progress. The stored set of commands has been termed a motor tape (Hoyle, 1964) or a motor score (Wilson, 1968a).

Of these possibilities the last, that of endogenous motor pattern generators, has been documented most thoroughly. Particularly pertinent examples here come from studies of insect flight and sound production. In locusts and moths the basic pattern of commands to wing muscles during flight is not altered by total or partial deafferentiation indicating that there is an endogenous program which produces the appropriate command patterns (Wilson, 1961; Kammer, 1967). Fixing the wings, changing the wing loading, or total deafferentiation does not essentially change the muscle activity patterns of crickets attempting to sing, again indicating that the motor activity results from an endogenous pattern generator (Kutsch and Huber, 1970; Bentley, 1969b). There are several reasons for believing that the activity patterns of warm-up and song in *N. robustus* also result from endogenous mechanisms which generate motor output patterns. Warm-up occurs with the wings folded in the normal resting position and with no obvious movement of the wings or other thoracic structures. Thus sensory information about wing position is not available for reflex arcs or for comparison with sensory templates. It is possible that receptors measuring muscle tension or deformation of the thoracic exoskeleton could give timing cues but the organization of warm-up activity, with simultaneous contraction of antagonistic muscles, seems designed to minimize thoracic movement and makes it seem unlikely that proprioceptors play a necessary role in the generation of the pattern. The short cycle length during singing poses problems for reflex chain or sensory template models for there is insufficient time during a cycle to initiate and process sensory information affecting later parts of the cycle. This does not completely rule out schemes requiring sensory input, however. For example, it could be proposed that singing results from a peculiar reflex chain in which sensory input indicating wing closure triggers not the next wing opening but the wing opening one or more cycles later. The most compelling evidence for an endogenous pattern generator is the observation that a set of synergistic muscles can skip one or several consecutive cycles without disrupting the rhythm, the next muscle action potential following a skipped cycle appearing just where it would be predicted on the basis of the intervals preceding the skip. Were the pattern generated by a reflex chain, a skipped cycle should terminate the rhythm; were a sensory template involved, a skipped cycle would result in an unusually large discrepancy between actual and expected sensory input and hence an altered motor output. Neither is the case. Thus singing and probably warm-up as well in *N. robustus* are members of a growing list of activities controlled by endogenous generators of motor patterns.

The mechanism generating the singing pattern

In our speculations about the mechanisms producing the motor output patterns of singing we will assume that information flows along a sequential set of elements. An element may be a single neuron or a group of neurones which modifies the information in a particular way. We will also assume that feedback from distal to proximal points in the command chains does not play an essential role. Because of the short cycle length there is little time for processing feedback information and, pragmatically, a seemingly satisfactory model can be proposed that does not require feedback. Particular attention will be given to irregularities sometimes seen in the singing pattern, specifically the alternation between long and short intervals. The mechanism generating this alternation is not known. Intuitively it seems possible that a neuron receiving a regular, phasic input could respond with pulses in alternating intervals if: (1) the intervals between inputs were close to the neurone's refractory period, and (*2*) there was post-firing depression in the neurone, the effects of which lasted more than a single cycle and the magnitude of which was greater the shorter the interval between firings. Thus if an interval happened to be shorter than usual, the excitability after firing would be lower and the next interval longer than average. Conversely after a long interval there would be less depression so the next interval would be short. Of course one can offer schemes in which a multicellular network responds with alternating intervals. But whatever way such patterns are produced, they are useful as markers, for when introduced into the command chain they will be propagated along the chain unless they reach some portion which smoothes out irregularities. As an example of the use of alternation as a marker consider the pattern shown in Figure 8C. Here the alternation is confined to the closer channel. This indicates that there is a point of lability at which alternation can originate distal to portions of the command chain shared by the opener and closer channels.

The following seem likely components of the central mechanism generating the activity patterns of singing. The scheme proposed is summarized in Figure 13. (1) Each forewing muscle is innervated by a set of motorneurones, some or all of which are involved in singing. Judging by the innervation patterns of other insects each motorneurone probably innervates only one muscle (*e.g.*, Bentley, 1970). This is partially confirmed in *N. robustus* by the unit activity sometimes recorded

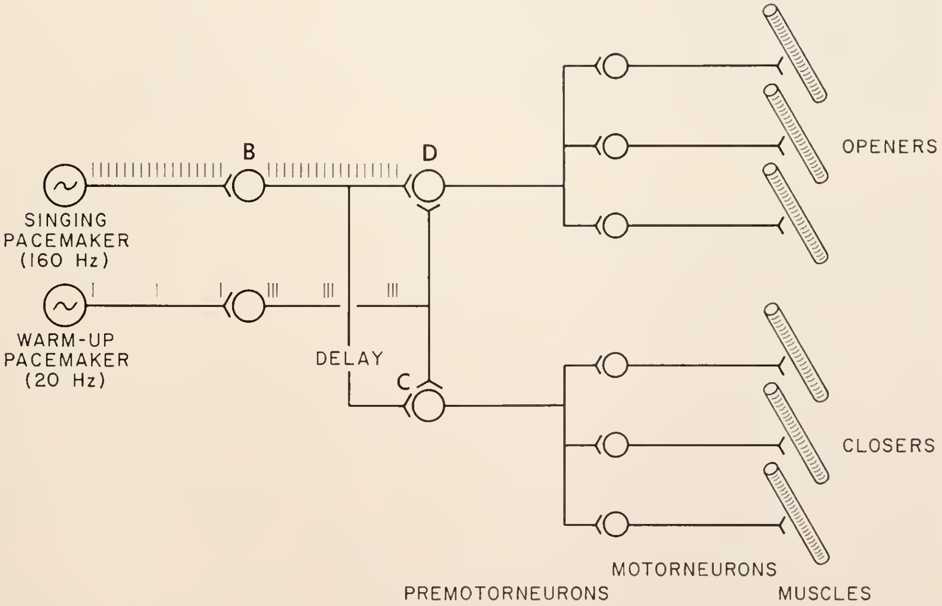

FIGURE 13. The model proposed for the generation of singing and warm-up motor patterns. The short vertical bars to the left are the spike patterns expected from those parts of the circuit. The singing and warm-up pacemakers may be mutually inhibitory since they do not simultaneously contribute to the output. B, C and D indicate the points in the circuit at which alternation might originate to create the interval patterns shown in Figures 8B, C and D, respectively.

in only one of several channels. (2) There is probably an interneurone or a group of tightly coupled interneurones which can simultaneously activate those opener motorneurones involved in singing and, similarly, an interneurone or group of interneurones which can simultaneously activate the closer motorneurones. For convenience these elements will be called the opener premotorneurone and the closer premotorneurone, even though it is recognized that each may consist of several nerve cells arranged serially or in parallel. The presence of such elements may be inferred from the strict synchrony in action potentials from all synergistic muscles during singing. Ordinary synaptic couplings between synergistic motorneurones, with their attendant delays, would not seem adequate for the coincident firing. Electrical coupling between motorneurones like that which insures synchronous firing among electromotorneurones in a weakly-electric fish (Bennett, Pappas, Aljure, and Nakajima, 1967) is a possible mechanism for simultaneous firing. Electrical coupling between motorneurones has been found in insects (Kendig, 1968; Bentley, 1969a). If there is electrical coupling between motorneurones in *N. robustus* it must not be very tight coupling for sometimes during warm-up and at the onset of singing one motorneurone can fire independently of its synergists. Further, the two tergocoxal muscles which are synergists during singing are probably antagonists during walking. If singing and walking involve the same motor units these must be capable of independent activity. For these reasons we suggest that the synchrony between synergists is a result of common excitatory input from premotorneurones, possibly augmented by electrical coupling. Interval alternation affecting all muscles in the synergistic group but not the antagonists of the group must begin at the premotorneurone level. (3) The opener and closer premotoneurones are driven from a common source which fires at the singing frequency but there is a fixed delay of approximately 3 msec introduced into the closer portion of the command chain. The presence of a common driver for both opener and closer premotorneurones is indicated by the activity pattern shown in Figure 8b. Similar patterns were recorded from two other animals. In these records both the openers and the closers had alternating long-short intervals and the closer interval lengths were nearly exactly the same as those of the opener intervals in which they began. The result is that irregularities in the opener channel are precisely reflected in the closer channel one-half cycle later. These irregularities must arise in some part of the command pathway common to both opener and closer premotorneurones. The reason for postulating a fixed delay in the closer pathway is obvious from Figure 8B. Although the opener and closer intervals fluctuate considerably the interval between opener and closer muscle action potentials is essentially constant. A fixed delay could be achieved in several ways. It might result from a single additional synapse inserted into the closer chain. Or the input to the opener premotorneurone might simultaneously inhibit the closer premotorneurone, the closer premotoneurone then firing as it escapes from inhibition several milliseconds after the opener premotorneurone has fired. The latter is essentially the explanation offered by Bentley (1969b) to account for the fixed delay between opener and closer muscle firing during cricket stridulation. This delay, like that in *N. robustus,* is of fixed duration although it is considerably longer than that found in the katydid (sixteen as opposed to three milliseconds).

Were it not for the alternating interval pattern recorded from one animal, that of Figure 8D, the fixed delay between opener and closer activity could be ascribed to transit time across an excitatory synapse from the opener to the closer premotorneurone. In this record there is alternation in the opener but not the closer channel. To interpret this one needs to know if the alternation occurred in all opener muscles, and therefore arose at the premotorneurone level, or if the alternation was restricted to one opener channel and therefore originated at the motorneurone level. Although only one opener muscle was monitored in this preparation it is reasonably certain that the alternation occurred in a number of opener muscles. With the method used some part of the recorded signal results from activity in muscles near the one containing the electrode. The contributions from nearby muscles should appear at different times in the main signal if the muscle being monitored were changing its phase relations to other openers. But the potentials actually recorded had essentially the same shape during each cycle, suggesting that synchrony between openers was maintained and that all openers were responding with similar alternating intervals (see the lower muscle record of Figure 9B3 which was from this animal). Further, the electrical record from the closer muscle of this animal had small spikes between the major closer spikes. The small spikes result from electrical pick up of opener activity. The intervals between the small spikes too alternated between long and short values. Since the small spikes are probably crosstalk resulting from the summed activity of several openers this again indicates that a number of opener muscles took part in the alternation and that alternation originated in the opener premotorneurone. But alternation did not occur in the closer muscle, indicating that the closer premotorneurone is not directly driven by the opener premotorneurone. Thus the portions of the command chain which are common to the opener and closer muscles must precede the premotorneurone level.

Generation of warm-up activity

Thoracic warming by muscular activity, similar to that seen in *N. robustus* before singing, has been found to precede flight in a number of insects (see Kammer, 1968; McCrea and Heath, 1971; and references therein). The strategy of warm-up appears to be to produce heat by contraction of wing musculature without producing full-scale wing movements. Wing movements before appropriately high thoracic temperatures are reached are likely to be ineffective (the power available from cool muscles may not be sufficient to maintain flight; a low pulse frequency in song may not be seductive to females) and they can attract the attention of predators. Wing movements during warm-up are minimized in several ways. In animals with asynchronous muscles the wings may be folded and mechanically uncoupled from the musculature during warm-up (Leston, Pringle and White, 1965). In animals with synchronous muscles the phase relations between flight antagonists are altered during warm-up. In some moths and butterflies, as in *N. robustus*, all units are activated nearly synchronously during warm-up. In other moths there are phase changes so that some units fire synchronously with normal antagonists and in antiphase to normal synergists (Kammer, 1968, 1970; Hanegan and Heath, 1970). Kammer (1968) raised the possibility that neuronal circuitry generating motor patterns may be temperature sensitive so that the transition between phase relations characteristic of warm-up to those of flight are an automatic consequence of rising temperature. In *N. robustus* warm-up activity completely stops and there is a delay of variable duration before singing begins. Similarly there is sometimes a delay between warm-up and the onset of flight in the moth *Hyalaphora cecropia* (Hanegan and Heath, 1970). The occurrence of a delay suggests that the transition from warm-up to singing or flight involves more than a temperature-sensitive transition between two output patterns from a single generator. A model in which temperature receptors and higher order integrative centers control the output of separate warm-up and flight pattern generators is proposed by Hanegan and Heath (1970).

The overall synchrony in muscle activity during warm-up suggests that both premotorneurones are then being driven by the same source. One possibility for the warm-up pattern would be an element firing at 10–20 per second which triggers short bursts in a follower which in turn drives both opener and closer premotorneurones (Fig. 13).

In summary, the model proposed for warm-up and song in *N. robustus* contains two central pacemakers, one which provides the singing frequency of 150–200 per second and one which fires at 10–20 per second giving the burst frequency during warm-up. There are probably at least three neuronal elements in series between the forewing muscles and the pacemakers; motorneurones, premotorneurones, and an element which activates the two premotorneurones.

Comparison with some related behaviors

Mechanisms of sound production in insects have been most extensively investigated in crickets. Singing is considerably more complex in the cricket species which have been studied than in *N. robustus*. Each species produces several distinct songs and in each song the muscle action potential patterns contain a number of frequency components rather than just one as is the case in the only known song of *N. robustus*. For example, in the calling song of crickets, which is functionally equivalent to the song of *N. robustus*, the singing muscles may fire several times per wing stroke and the sound pulses produced by individual wing strokes are grouped in chirps with quiet periods in between (Ewing and Hoyle, 1965; Bentley and Kutsch, 1966; Bentley, 1969b, Kutsch, 1969). On the basis of intracellular recordings from motorneurones and interneurones in singing crickets Bentley (1969b) has proposed that the chirp rhythm is determined by a central oscillator while activity patterns within a chirp result from excitatory interactions between the motorneurones, possibly mediated in part by interneurones driven by the motorneurones. The models proposed for singing in crickets and *N. robustus* differ in detail but they do contain an essential similarity; in each there is assumed to be a central oscillator which operates without feedback from the motorneurones and whose output, through interneurones, triggers the motorneurones.

Muscle activity patterns during insect flight have been extensively studied by D. M. Wilson and his colleagues (for a review see Wilson, 1968b). In the locust, *Schistocerca*, the basic motor pattern is endogenously generated but sensory input can alter the frequency and, through reflexes which are slowly acting with respect to the flight frequency, modify the power output on the two sides to insure flight stability (Wilson, 1961, 1968a). In *Schistocerca* the phase relations between antagonists remain constant over a rather wide frequency range (Waldron, 1967);

this is unlike the singing pattern in *N. robustus* and crickets (Bentley, 1969b) where the time between opener and closer firings is relatively constant and therefore the phase of closer firing changes with changes in the opener-opener interval. To meet the criterion of neuronal economy the flight pattern in locusts has been explained largely on the basis of interactions between neurones at one level, possibly the motorneurones themselves (Wilson, 1966). While the rhythm and phasing between elements of the flight system can be explained on the basis of excitatory and inhibitory interactions between elements at a single level, a hierarchical arrangement of participating neurones, like that proposed here for *N. robustus,* cannot be ruled out. Indeed, as Wilson (1968b) points out, a hierarchical organization is an appealing way to account for the ability of motorneurones to participate with different activity patterns in several behaviors.

SUMMARY

1. During stridulation the forewings of *Neoconocephalus robustus* are rubbed against one another at a frequency of 145–212 per second. Despite the high frequency the forewing muscles are synchronous muscles; each contraction is preceded by a muscle action potential.

2. The direct flight muscles of the mesothorax are wing openers during singing; the indirect flight muscles are wing closers. The sound pulse is produced on the closing stroke of the wings.

3. Singing is preceded by warm-up during which all forewing muscles are activated synchronously. In early warm-up the muscles are activated in short bursts, often at a regular frequency. Later warm-up activity is continuous. Muscle activity stops briefly at the transition from warm-up to singing.

4. Muscle activity patterns during singing indicate that the motor output results from an endogenous pacemaker which fires at the singing frequency. There are probably at least three neuronal elements in series between the pacemaker and the forewing muscles. The phasing between opener and closer muscles results from a fixed delay of approximately 3 msec between opener and closer portions of the command chain.

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HIGH FREQUENCY MUSCLES USED IN SOUND PRODUCTION BY A KATYDID. II. ULTRASTRUCTURE OF THE SINGING MUSCLES[1]

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Males of the tetigoniid *Neoconocephalus robustus* stridulate by rubbing the edges of the forewings together to produce an apparently continuous and high pitched note. The accompanying paper by Josephson and Halverson (1971) has shown that one sound pulse is produced each wing cycle and that the frequency is 145–212 per second. While wing stroke frequencies even much in excess of this have been recorded from insects with asynchronous flight muscles (Smith, 1965a), frequencies in excess of 100 Hz are uncommon in the lower insect orders (Sotavalta, 1947).

The present study was undertaken therefore because it was of considerable interest to determine whether the flight muscles were of the synchronous or of the asynchronous type. The latter type are present only in the orders Coleoptera, Diptera, Hymenoptera, Hemiptera and probably the Thysanoptera (Pringle, 1967). Thus if the muscles were of the asynchronous type it would be a new finding for the Orthoptera and if they were of the synchronous type they would be the fastest described synchronous insect flight muscles and amongst the fastest synchronous muscles in the animal kingdom.

The ultrastructural differences between synchronous and asynchronous (fibrillar) muscles are now well recognized (Smith, 1965b, 1966). One of the most striking features of fibrillar muscle fibers is the almost complete absence of the sarcoplasmic reticulum, which contrasts strongly with the findings in fast neurogenic muscles, from both vertebrates and invertebrates (Fawcett and Revel, 1961; Revel, 1962; Fahrenbach, 1963; Reger and Cooper, 1967), in which the sarcoplasmic reticulum is very well developed, culminating in the extreme condition of a fast acting lobster muscle in which the sarcoplasmic reticulum occupies 75% of the total muscle volume (Rosenbluth, 1969). The structural features therefore of the stridulating mesothoracic muscles of *N. robustus* are examined in relation to other fast acting muscles in an attempt to evaluate those structural features which enable the muscle to function at such high frequencies. They are compared with the flight muscles of the metathoracic segment, which do not participate in stridulation, and with the flight muscles of male *N. ensiger,* a species which overlaps in range with *N. robustus* but stridulates with a frequency of only 10–15 Hz (Heath and Josephson, 1970).

MATERIAL AND METHODS

Males of *Neoconocephalus robustus* and *N. ensiger* were collected from salt marshes in the vicinity of Woods Hole, Massachusetts and kept in outdoor cages until required, as described in Josephson and Halverson (1971). The head and

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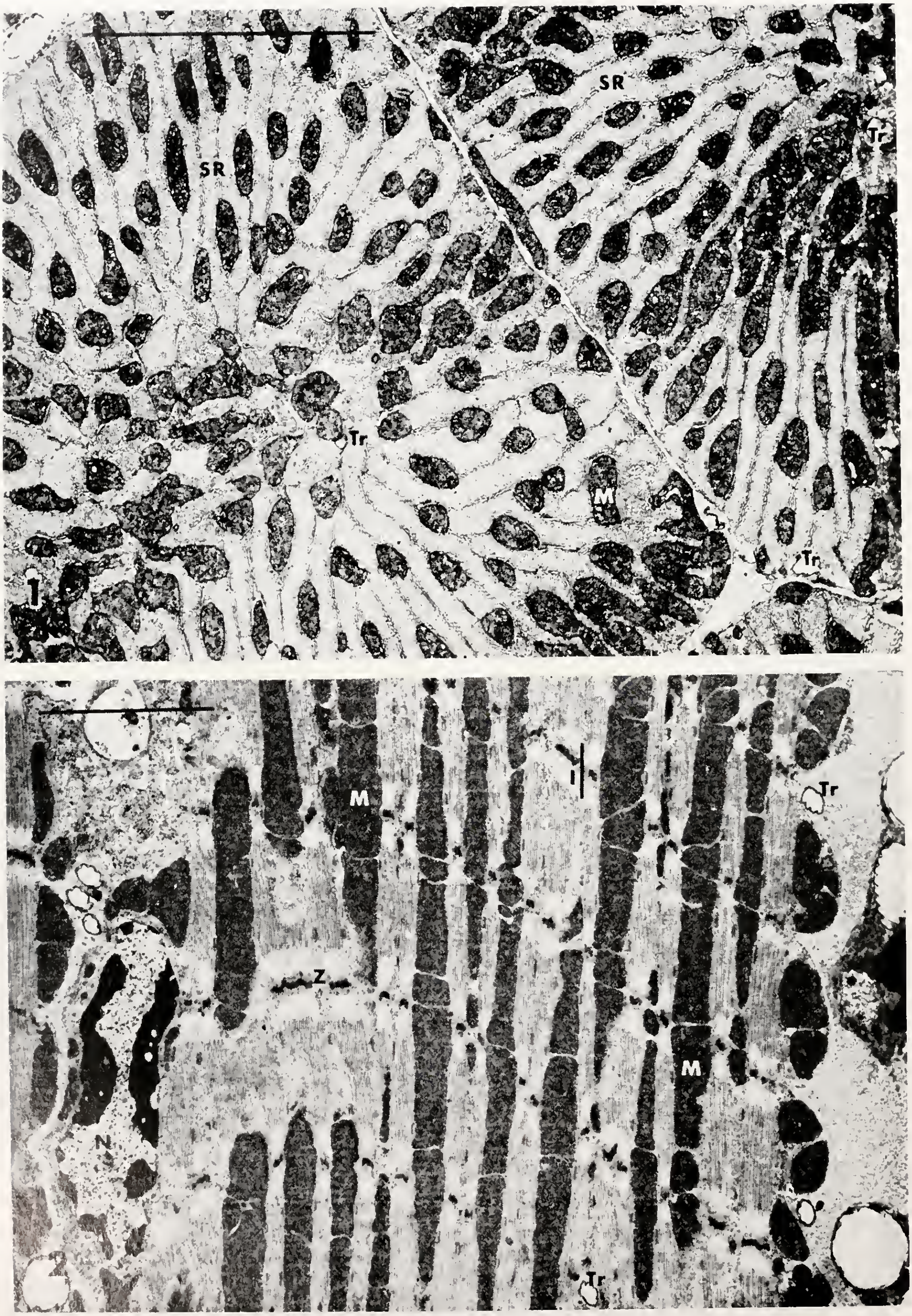
abdomen were removed and the segment of gut pulled out from the thorax. Fixative was either pipetted through the central cavity of the thorax and the whole thorax immersed in fixative or the terga and sterna of the thoracic segments were cut along the mid-line and the halves were immersed in fixative. By either method the attachments of all the muscles to the exoskeleton remained intact. The fixative employed was 3% glutaraldehyde in 0.1 M Millonig phosphate buffer with 3% sucrose at pH 7.4 for 2 hrs at 4° C. The tissues were washed and stored for transport in 5% sucrose in the phosphate buffer at pH 7.4 and 4° C. On the day following fixation the tissues were transported by air in insulated containers to the United Kingdom. On arrival they were post fixed in 1% OsO_4 in 0.1 M phosphate buffer at pH 7.4 and araldite embedded. Samples from the edges of the muscle blocks only were taken for embedding. Duplicate specimens were later taken from insects flown live to the United Kingdom from Woods Hole. Sections were stained with uranyl acetate and lead citrate and examined in an AEI EM6B electron microscope.

For stereometric analysis of the quantity of sacroplasmic reticulum present and the volume of the fibers occupied by mitochondria the combined point count and intersect incidence method of Freere and Weibel (1967) was used. The formulae employed were those for volume estimation (mitochondria and sarcoplasmic reticulum) and surface density (sarcoplasmic reticulum) and the data were obtained by superimposing a cellulose acetate grid with 84 lines and 168 points on electron micrographs of suitable magnification.

Description of fibers

Each of the muscle groups of the stridulating (mesothoracic) flight muscles of *N. robustus,* direct (basalar and subalar), indirect (tergosternal, tergocoxal 1 and 2, pleurotergal and oblique tergal) and median dorsal longitudinal synergists (see Josephson and Halverson, 1971) consists of small fibers of only 10–25 μm in diameter which are arranged around the large central tracheae. Each fiber has peripheral nuclei and the ribbon-like fibrils are radially arranged. Such "radial lamellar" packing with peripheral nuclei is typical of the flight muscles of the Orthoptera (Tiegs, 1955; Pringle, 1965). The metathoracic fibers and meso- and metathoracic fibers of *N. ensiger* are also of this type.

The fibers are ovoid or polygonal in cross section and the strap-like myofibrils are arranged in radial fashion interspersed with numerous large mitochondria (Fig. 1). While the arrangement lacks the almost geometrical precision of some other described flight muscles of the radial lamellar type (Smith, 1962), the form is extremely compact. The mitochondria are subcylindrical in shape, ovoid in cross section and with a rectangular outline when sectioned longitudinally. They are often found packed end to end in rows between fibrils (Fig. 2). Due to the large number of tightly packed cristae the mitochondria are very electron dense. The cristae have a characteristic appearance, often being found tightly packed in sectors of concentrically packed, curved cristae and have the dense appearance and ill-defined cristal detail now recognized as typical of primary fixation with glutaraldehyde (*e.g.,* Ashhurst, 1967). Two configurations were frequently found, normal (Figs. 2, 3, 8) and vesiculated (Figs. 5, 7). In the latter many of the cristae in each mitochondrion of particular fibers had a dilated appearance and it is not clear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Figures 1–2.

whether these mitochondrial forms represent configurational changes due to different configurational states (Hackenbrock, 1968) or are fixation artifact such as Stoner and Sirak (1969) have described.

Using stereometric methods (Freere and Weibel, 1967) the mitochondrial volume in the mesothoracic flight muscles of male *N. robustus* was found to be 44% of the total fiber volume (Josephson and Elder, 1968). Although the mitochondria in the metathoracic fibers are of the same appearance as those described above they are significantly fewer in number (Fig. 3).

The sarcomeres are short (approximately 3 μ) with narrow I-bands. The sarcomeres of the median dorsal longitudinal muscles are significantly longer than those of the other direct and indirect flight muscles (approx. 4.2 μ). The margins of the A-bands are rather ill-defined and M-lines are absent. The thick filaments of the A-band are approximately 2.4 μ in length (3.3 μ in the median dorsal longitudinal muscles). In all fibers the myosin filaments are approximately 16 nm in diameter with a center to center spacing of approximately 48 nm. The thin filament margins in the A-band are also not in perfect register and thus in longitudinal sections an H-band is difficult to distinguish. In transverse sections of relaxed muscle the presence of the H band is recognizable by the absence of thin filaments (Fig. 5). Transverse sections show the thick filaments disposed in hexagonal array and each thick filament is surrounded by an orbit of six thin filaments. Each thin filament is located equidistant between adjacent pairs of thick filaments, as in many other insect flight muscles (Spiro and Hagopian, 1967), which gives a thin to thick ratio of 3:1 (Smith, 1966). In contracted fibers orbits of twelve thin filaments around each thick filament are found in the central regions of the A-bands due to "double overlap" of the thin filaments (Fig. 4).

Glycogen granules are found mainly near the Z-line regions between fibrils but they also occur in rows between thin filaments in the I-band regions within fibrils (Figs. 11, 12).

The sarcoplasmic reticulum (SR) is very well developed, particularly in the mesothoracic flight muscles of *N. robustus* (Figs. 4–6). In sections which lie for some distance in interfibrillar spaces the form of the SR is seen to be that of a continuous network of interconnected tubules forming perforate curtains which completely surround each fibril (Figs. 6, 9, 10). In many locations overlapping has occurred so that between fibrils there may be as many as four or more layers of SR tubules (Figs. 4–6). A layer of SR is found between the myofibrils and the mitochondria so that all fibrils are completely surrounded by at least one thickness of SR membranes. The stereometric estimation shows that the SR occupies

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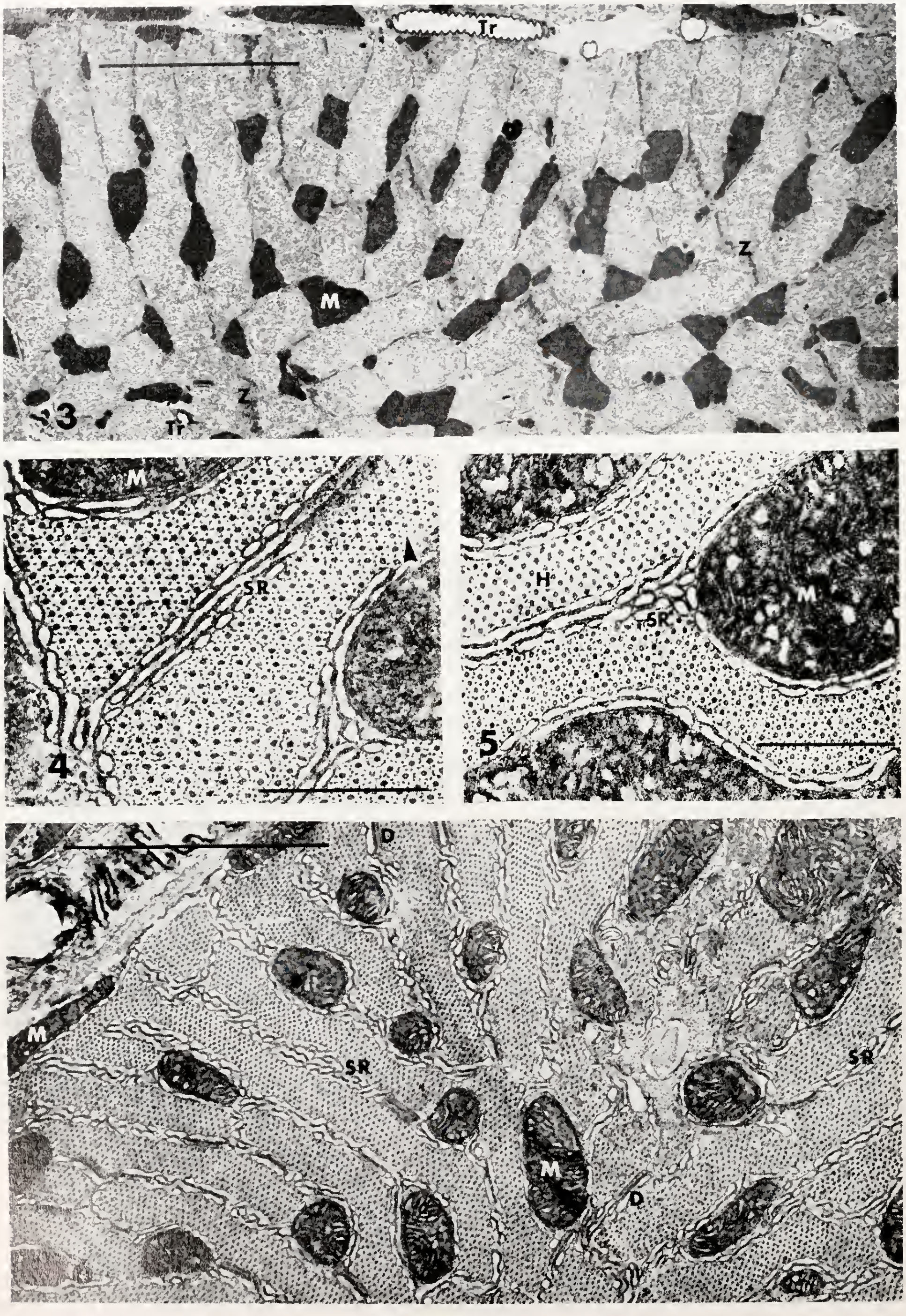
FIGURE 1. *N. robustus,* transverse section of the mesothoracic median dorsal longitudinal muscle shows the form of the radially arranged narrow fibrils and the numerous, large mitochondria. Well developed sarcoplasmic reticulum surrounds the fibrils and tracheae running longitudinally in the center of the fiber are seen. Abbreviations are: D—Dyad, G—Glycogen granules, H—H band, I—I band, M—Mitochondria, N—Nucleus, SR—Sarcoplasmic reticulum, T—T system cleft, Tr—Tracheole, Z—Z line; scale line = 5 μ.

FIGURE 2. *N. robustus,* longitudinal section of the mesothoracic tergocoxal 1 muscle. The nodular profile of a fiber, caused by the peripheral location of mitochondria opposite the A-bands in many sarcomeres is seen. Most of the mitochondria have a rectangular profile and pack in rows between the fibrils. Numerous tracheae and the peripheral nuclear location are also seen. In this extended region of the fiber the I-band is prominent; abbreviations as in Figure 1, scale line = 5 μ.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Figures 3–6.

approximately 19% of the total fiber volume and has a surface area of approximately 14 μ^2 per μ^3 of fiber volume. Stereometric analyses of the fibers from *N. ensiger* or of the metathoracic fibers of *N. robustus* have not been made but it can be seen that the development of the SR of the mesothoracic fibers of *N. ensiger* approaches that in *N. robustus* (Fig. 7) while the metathoracic flight muscles of both species have the same pattern of SR organization but clearly less well developed than in the mesothoracic segments (Figs. 3, 8).

T-system clefts (30 to 40 nm by 120 to 360 nm) invaginate at the level of overlap of the thick and thin filaments (Figs. 8, 9), *i.e.*, two invaginations per sarcomere length. At regular intervals along each sarcomere of each fibril dyads are formed by the close association of a T-tubule and an overlying cysternal plaque of the SR. These are located in the A-band at the regions of overlap of thick and thin filaments (Fig. 9). The T-system invaginations are not of uniform dimensions along their length. Where dyads are formed the T-system clefts expand to form periodic plaques up to 600 nm across (Fig. 10). Electron dense granular material is frequently seen in the T-tube lumina of the dyads. The spacing of these granules appears to be fairly regular at about 38 nm (Fig. 11). Since the form of the T-system is that of a flattened cleft rather than that of a tubule with a cylindrical cross section, invaginations are more readily found in transverse sections (Fig. 8) than in longitudinal sections (Fig. 9). In the mesothoracic fibers of both *N. robustus* and *N. ensiger* the presence of peripherally located mitochondria gives these fibers a nodular profile in longitudinal section (Fig. 2) which frequently causes the T-system invaginations to be displaced longitudinally to such an extent in some locations that the invaginations are initially found to run longitudinally to reach the location of dyad formation at the region of thick and thin filament overlap (Fig. 12).

Periodic large diameter invaginations of the sarcolemma, at the level of the Z-lines, allow tracheoles to penetrate the full radius of the muscle fibers and one or more tracheoles are frequently found running longitudinally at the center of each fiber (Figs. 1, 3). At the periphery of the fibers the Z-lines attach to the plasma membrane by hemi-desmosomes. Seen in longitudinal section the Z-lines are rather broad, ill-defined, electron dense areas (Figs. 2, 12). In contracted muscle the

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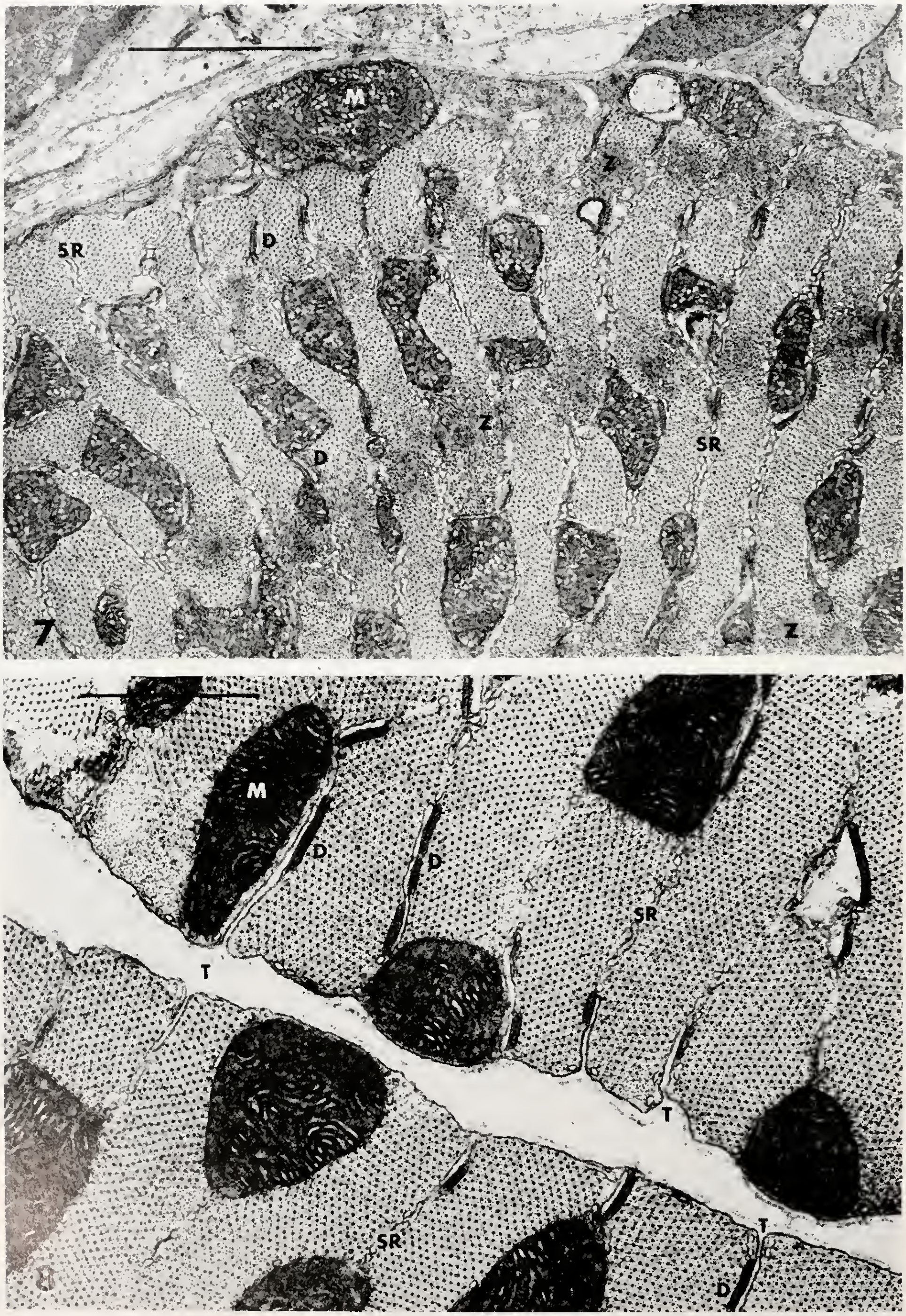
FIGURE 3. *N. robustus,* transverse section of the metathoracic median dorsal longitudinal muscle. The fibril organization is radial lamellar but the fibrils are wider, the mitochondria are less numerous and the SR is less well developed than in the singing muscles; abbreviations as in Figure 1, scale line = 5 μ.

FIGURE 4. *N. robustus,* transverse section of the mesothoracic first tergocoxal muscle. The normal insect flight muscle filament packing pattern with six thin filaments surrounding each thick filament and with the thin filaments equidistant between adjacent thick filaments is seen at the top of the figure (*e.g.,* arrow). At the bottom of the figure double overlapping of thin filaments giving orbits of up to twelve around each thick filament is apparent. The well developed SR and vesiculated mitochondria are again prominent; abbreviations as in Figure 1, scale line = $\frac{1}{2}$ μ.

FIGURE 5. *N. robustus,* transverse section of the mesothoracic first tergocoxal muscle. The narrow strap-like fibrils are surrounded by layers of SR tubules and the H-band is apparent in this field. The mitochondria have a vesiculated appearance (see text) and the myosin filaments appear to be hollow; abbreviations as in Figure 1, scale line = $\frac{1}{2}$ μ.

FIGURE 6. *N. robustus,* transverse section of the mesothoracic median dorsal longitudinal muscle shows the extensive development of the SR tubules which ensheath the ribbon-like fibrils. In most locations there are at least two layers of SR and in some, up to five; abbreviations as in Figure 1, scale line = 2 μ.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Figures 7–8.

thick filaments penetrate some way into the electron dense Z-line material and while no myosin filaments have been seen completely penetrating a Z-line and nothing approaching the supercontraction described by Osborne (1967) and others has been observed, the situation in the katydid flight muscles appears to be very similar to that described by Hagopian (1970) in chick muscle.

Discussion

The evidence of Heath and Josephson (1970) indicates that the metabolic activity of the mesothoracic flight muscles of *N. robustus* is very high during stridulation. The more metabolically active a tissue is, the greater the number of mitochondria it contains and amongst skeletal muscles it is the tonic muscle more or less continually active in postural activity, and the phasic muscles specialized to produce the fastest speed which have the highest number of mitochondria (Hoyle, 1969). Josephson and Elder (1968) found the high value of 44% of the total fiber volume occupied by mitochondria in the flight muscles used in stridulation by *N. robustus*. The figure is similar to that calculated by Smith (1961) for *Aeshna* flight muscle, another fast acting synchronous flight muscle.

Amongst fish very fast acting muscles with a mechanical response of up to 200 Hz have been described by Fawcett and Revel (1961). Revel (1962) has described a muscle with a similar contraction rate amongst mammals. The ultrastructure of some fast acting crustacean muscles has been described by Farenbach (1963, 1964) and Rosenbluth (1969). Sotavalta (1947) measured the wing stroke frequency of a large number of insects and recorded frequencies up to several hundred cycles per second and even in excess of 1000 Hz. All such very high frequencies have been found hitherto amongst the five insect orders with asynchronous muscles (Pringle, 1967). Following Pringle's (1949) work it was appreciated that these muscles represent a special category of muscle in which the contraction frequency may be far in excess of the rate of efferent impulses. It was shown by Boettiger (1951) that the oscillatory frequency of these muscles depends upon the load and not upon the frequency of neuronal stimulation.

By contrast the synchronous flight muscles found in other insect orders, including the Orthoptera, although providing examples of fast acting muscles, rarely exhibit frequencies above 100 Hz (Sotavolta, 1947), although Josephson and Halverson (1971) have predicted that the very fast wing stroke frequency (280 Hz) found in stridulating *Orocharis gryllodes* (Walker, 1969) will prove to be powered by synchronous muscle. Ultrastructural studies of synchronous muscles from insects with relatively high wing stroke frequencies have been made by Auber (1967), Reger and Cooper (1967) and others. With a mechanical response of 150 to 200 Hz the muscles described in the present study appear to be the fastest synchronous

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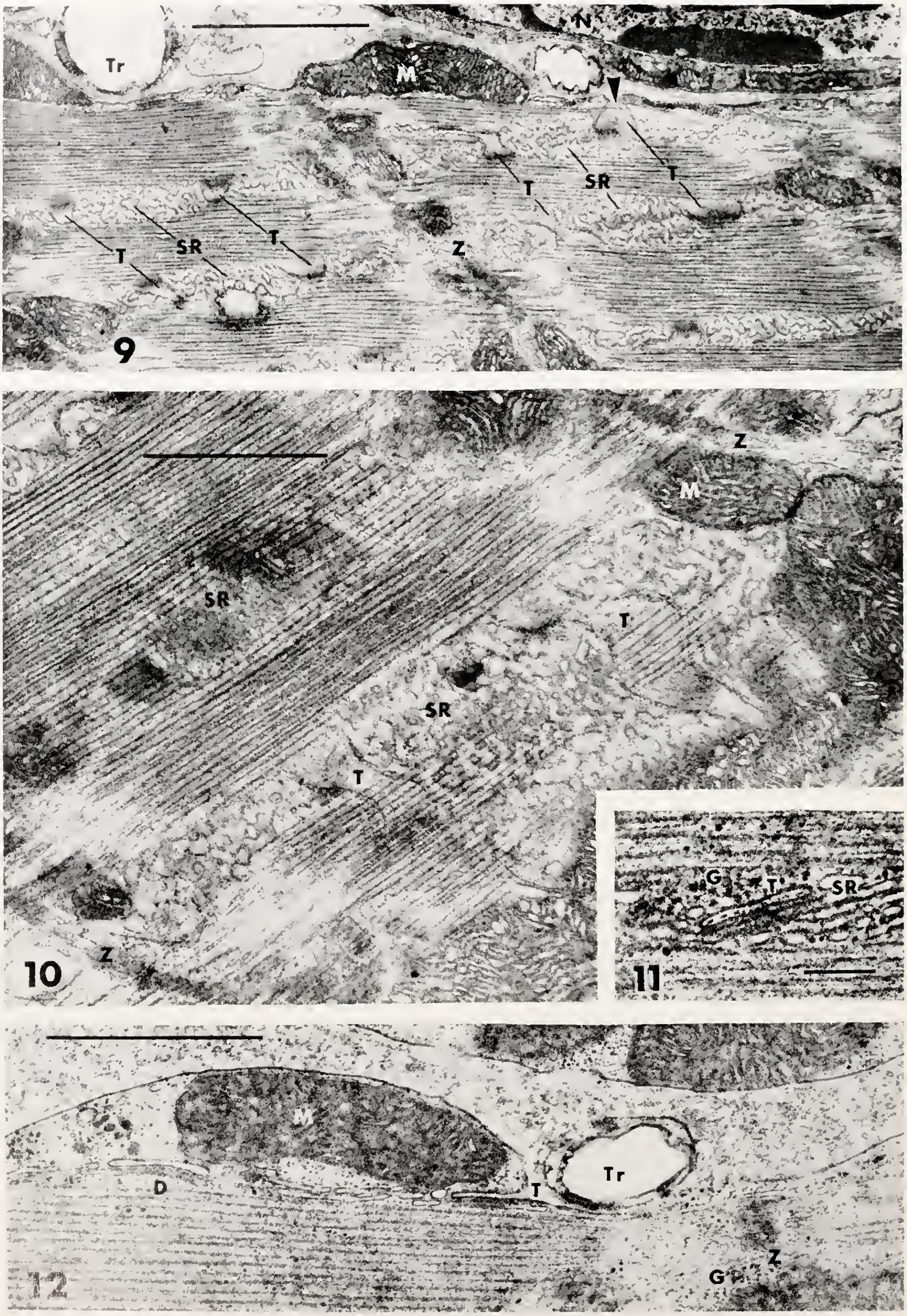
Figure 7. *N. ensiger*, transverse section of the mesothoracic first tergocoxal muscle. The singing muscles of this species have a very similar appearance to those of *N. robustus*. Mitochondria are numerous and the well developed SR surrounds the narrow fibrils; abbreviations as in Figure 1, scale line = 2 μ.

Figure 8. *N. ensiger*, transverse section of the metathoracic median dorsal longitudinal muscle. In this species also the metathoracic fibers have broader fibrils and a less well developed SR than those of the mesothorax. In this field, from the region of overlap of thick and thin filaments, a number of T-system invaginations are seen to form dyads with elements of the SR containing electron dense material; abbreviations as in Figure 1, scale line = 1 μ.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FIGURES 9–12.

flight muscles so far described and it is of value to compare their structure with that of the asynchronous type and with other known fast acting vertebrate and invertebrate muscles.

The structure of insect fibrillar muscles is well known from the papers of Tiegs (1955), Smith (1961, 1966) Shaffiq (1963, 1964), Ashhurst (1967), and others. The fibers are usually of large diameter (100-200 μ) with well spaced cylindrical fibrils, 1–3 μ in diameter. The transverse tubules are variably placed but frequently at the level of the M-line. The sarcoplasmic reticulum is strikingly reduced and limited to the cisternae of the dyads, Z-line vesicles and a few other irregularly placed tubules. The sarcomeres are short, the I-band is narrow and an M-line, unusual in insect muscle, is present. The tapered ends of the myosin filaments are attached to the Z-band material. Numerous dense and irregularly shaped mitochondria pack much of the space between the fibrils.

In most of these respects the muscle in the present study differs strikingly from the fibrillar muscle characteristics and corresponds to other described synchronous insect flight muscles of the radial lamellar type. Thus the fibers are only some 10–25 μ in diameter with ribbon-like fibrils only 200–400 nm wide arranged in radial fashion. Sarcoplasmic reticular tubules totally surround each fibril. Stereometric estimation shows that the vesicles occupy approximately 19% of the total fiber volume and have a surface area of some 14 μ^2 per μ^3 of fiber. T-tubes invaginate regularly opposite every A/I overlap region. Dyadic associations between the T-tubes and the SR membranes thus occur twice per short sarcomere length (3–4.2 μ) which, combined with the narrow fibril width, means that a very large number of T-tube invaginations are present. Calculation suggests that the incidence would be at least one T-tube invagination per μ^2 of muscle membrane. This compares with an incidence of approximately only 0.6 T-tube invaginations per μ^2 of membrane in frog sartorius muscle. The internal volume and surface area of the T-system will be much greater in frog sartorius, however, since the diameter of the latter is 5–10 times greater than that of the singing flight muscles. But estimated as volume or surface area per unit volume of fiber the quantities in these muscles may be similar, for little branching of the T-tubes was observed amongst the radially oriented, ribbon-shaped fibrils of *Neoconocephalus,* while Peachey and

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FIGURE 9. *N. robustus,* longitudinal section of the mesothoracic median dorsal longitudinal muscle. The invagination of a T-system cleft is contained within the thickness of the section (arrow). The plane of section passes obliquely through the SR ensheathing several fibrils. Two T-system clefts penetrate the fiber at the regions of overlap of thick and thin filaments in each sarcomere; abbreviations as in Figure 1, scale line = 2 μ.

FIGURE 10. *N. robustus,* longitudinal section of the mesothoracic median dorsal longitudinal muscle. The SR and T-system clefts are seen *en face.* Frequent branching of the SR tubules forms a perforate curtain which extends the length of the sarcomere. The T-system clefts are periodically expanded into plaques; abbreviations as in Figure 1, scale line = 1 μ.

FIGURE 11. *N. robustus,* longitudinal section of the mesothoracic first tergocoxal muscle at the region of dyad formation. The T-tubule frequently contains regularly spaced, electron dense granules in its lumen and the cisternal element of the SR contains finely granular, electron dense material; abbreviations as in Figure 1, scale line = 200 nm.

FIGURE 12. *N. robustus,* longitudinal section of the mesothoracic first tergocoxal muscle. Peripherally located mitochondria at the A-band regions appear to cause the longitudinal displacement of the T-system invaginations which therefore run longitudinally in their initial course in order to reach the region of dyad formation in the A-band. A dyad, probably formed in this way, is seen to the left of the figure; abbreviations as in Figure 1, scale line = 1 μ.

Schild (1968) have shown that the quantity of T-system present in frog sartorius muscle is some 30% greater due to branching than that estimated on the basis of incidence of surface invaginations at the Z-lines. While additional "Z-invaginations" (Peachey, 1968) are present primarily to conduct tracheoles into the depth of the fibers, no dyadic associations have been observed between them and elements of the SR such as have been observed in other arthropod fibers (Brandt, Reuben, Girardier and Grundfest, 1965; Cochrane, Elder and Usherwood, in press). No M-line is present and although the I-bands are narrow the thick filaments are not attached to the Z-line. Thus it is clear that these muscles have a structure typical of other radial-lamellar, synchronous muscles (*e.g.*, Smith, 1962, 1966).

Several of the ultrastructural features of the mesothoracic flight muscles of *N. robustus* can be interpreted as adaptations to a fast contraction-relaxation cycle. The very small diameter of these fibers will limit the length of the transverse tubular invaginations and therefore presumably minimize the time taken by the inward spread of excitation. Gonzalez-Serratos (1966), working with frog sartorius muscle, calculated that 8 cm/sec was the speed of inward spread of excitation at 20° C and that the velocity increased with temperature up to that point. Heath and Josephson (1970) have demonstrated that the functional temperature of the stridulating muscles in *N. robustus* is 36° C, some 10° C higher than ambient, and a value of 8 cm/sec for the inward spread of excitation might therefore be low for this insect muscle. However, if the figure of 8 cm/sec is adopted, this phase of the excitation/contraction coupling process in *N. robustus* muscles would take only 0.1 msec in fibers of 8 μ radius. The high incidence of T-tube invaginations in these muscles must represent an adaptation for achieving a rapid and even spread of excitation throughout the fiber. By contrast, the fast acting (100–130 Hz) remotor muscle of the lobster second antenna described by Rosenbluth (1969) forms a syncitium with connective tissue septa making incomplete longitudinal partitions at 50–100 μ intervals. T-tube-like extensions from the septa form dyads with cisternae of the SR at the region of overlap of thick and thin filaments. However, membrane depolarization is accompanied by Ca^{++} influx from the extracellular fluid (Mendelson, 1969), unusual in fast acting muscles, and Rosenbluth (1969) suggests that the entry of Ca^{++} ions from the extracellular fluid may directly trigger contraction or augment the action of Ca^{++} release from the terminal cisternae of the dyads.

Similarly the very narrow fibril width may well be an adaptation to minimize the time necessary to achieve an even distribution of Ca^{++} ions amongst the myofilaments by diffusion from the SR. Winegrad (1968) has shown that in frog skeletal muscle Ca^{++} is released from the SR terminal cisternae of the triad during activation and during relaxation is sequestered by the SR tubules surrounding the fibrils. Although the SR cisternal element of the dyad in synchronous flight muscle is not as well defined as the terminal cisternae of the vertebrate triad a similar mechanism may operate in these arthropod muscles. Thus although the length of the diffusion pathway in the singing flight muscles of *N. robustus* is not known exactly it must lie between approximately 1 μ and 100–200 nm. At maximum it would require only 1 msec for Ca^{++} ions to become evenly distributed over 1 μ (Ebashi and Endo, 1968) and a very small fraction of the 1 msec if the shorter distances are applicable since the time required for distribution depends upon the square of the linear dimension. Narrow fibril width (Fawcett and Revel, 1961; Revel, 1962; Smith, 1962) or a functional reduction in the distance between filaments and sarcoplasmic reticulum (Farenbach, 1963; Auber, 1967) is a normal feature of fast acting synchronous muscle of both vertebrates and arthropods.

The very large amount of SR present is a feature of the fast acting flight muscle and it seems probable that the greatly increased ratio of SR surface area to myofilament number (SR occupies approximately 19% of total fiber volume; filaments occupy approximately 36% of total fiber volume) is an adaptation to decrease the delay between stimulation and the release of that quantity of Ca^{++} ion necessary to initiate the mechanical response. Large quantities of SR are characteristic of other fast acting muscles (with the exception of insect asynchronous flight muscle) and it is well established that there is a relation in many muscles between speed of contraction and amount of SR present (Bendall, 1969; Hess, 1970; Cochrane *et al.*, in press). In the fast acting lobster muscle described by Rosenbluth (1969) the SR occupies three quarters of the total fiber volume. Relaxation is very rapid in these muscles (Mendelson, 1969) and the calculations of Van der Kloot (1969) have shown that the rate of Ca^{++} binding by the SR would be a function of the amount of SR surrounding the myofibrils even to the remarkable quantities present in the lobster remotor muscle. Such SR development greatly exceeds the SR volume estimated for the mesothoracic fibers in *N. robustus*. As Mendelson (1969) has pointed out, however, the large volume in the remotor muscle may be required in order that it can achieve contraction frequencies of 100 Hz at 16–18° C; some 20 C° lower than the muscle temperature in singing *N. robustus* (Heath and Josephson, 1970).

The singing flight muscle fibers of *N. robustus* have a short striation pattern (thick filament length of 2.4 μ and sarcomere length of approximately 3 μ) amongst arthropod muscles and even amongst flight muscles (Spiro and Hagopian, 1967). A short sarcomere length seems an obvious adaptation to fast action, since for a giver total fiber shortening, a muscle with short sarcomeres will undergo smaller percentage shortening of the sarcomere than one with long sarcomeres. In terms of the cyclical action of attachment and detachment of myosin bridges between thick and thin filaments, the shorter the sarcomere length, the fewer would need to be the cycles of cross-bridge make and break in a given time to produce a given total fiber contraction. In general, where sarcomere lengths vary, fast acting fibers have short sarcomere lengths (Spiro and Hagopian, 1967; Hoyle, 1969). Also the length by which insect flight muscles shorten is much less, as little as 5% in synchronous flight muscles *in vivo* (Weis-Fogh, 1956), than in most other muscles. This appears also to be the case in katydid flight muscles (R. K. Josephson, University of California, Irvine, personal communication). Further, the rate of rise of the twitch response may be greater in these muscles, as in other synchronous flight muscles, because of a relatively non-complaint series elastic component (Buchthal, Weis-Fogh and Rosenfalck, 1957; Pringle, 1965). Bárány (1967) found a 200 fold difference in ATPase activity amongst a variety of different vertebrate muscles; it would not be surprising to find high enzyme activities in *N. robustus* singing muscles also as a further adaptation to fast action.

The structural features of the singing flight muscles have been discussed above in an attempt to recognize those features which may be of importance in conferring the ability to respond at the very high frequencies found. There remains, however, the enigma of why the stridulating muscles of *N. ensiger*, which sings with the much lower frequency of 10–15 Hz (Heath and Josephson, 1970), are structurally very similar. Nor do the flight muscles of members of such well described groups as the Odonata (Smith, 1961, 1966) or species of the Lepidoptera with slow wing beat frequencies, such as *Pieris* or *Vanessa* (Auber, 1967; Reger and Cooper, 1967; Smith, 1962) or the locust *Schistocerca* (personal observations) differ strikingly in any of the essential features such as development of the T-system or sarcoplasmic reticulum or dimensions of the fibers and fibrils, despite probably unimportant differences in fiber architecture (tubular fibers in the Odonata and close packed fibers in the Lepidoptera). Yet they too operate, in flight, at a functional frequency approximately an order of magnitude slower than the stridulating flight muscles of *N. robustus*. The explanation may be that, as in *Schistocerca,* although the twitch is of brief duration (Buchthal *et al.,* 1957; Neville and Weis-Fogh, 1963) the wing frequency is limited by the long refractory period of the motor nerves which impose a maximum impulse frequency (Ewer and Ripley, 1963). The evidence of Josephson and Halverson (1971) shows that in singing *N. robustus* the flight muscles are driven at a frequency of 150–200 Hz by a central nervous pacemaker. It seems probable therefore that the much slower stridulation frequency in *N. ensiger* results not from any inability of the muscles to respond faster but from an imposed neural frequency. It may be concluded that in an Orthopteran already endowed with fast twitch muscles the greatest adaptation to high frequency wing movement has not been to increase the contraction/relaxation rate so much as in the provision of the central nervous pacemaker and refractory properties of the motor nerves. *N. robustus* may simply have exploited a potential which may already be present in many insect orders with synchronous flight muscles and the apparent lack of any striking structural difference between the stridulating muscles of the two species may therefore not be so surprising. In either species there is a greater difference between the mesothoracic singing muscles and those of the metathorax, used solely for flight, than there is between the singing muscles of the two species. The full significance of this adaptation to stridulation remains to be clarified.

Summary

1. The forewings of male *Neoconocephalus robustus* are rubbed together at frequencies of 150–200 Hz during singing. The ultrastructural organization of the mesothoracic flight muscles is typical of synchronous flight muscle.

2. The small diameter fibers (10–25 μ) are well supplied with tracheole branches which invaginate at the Z-lines. The radially arranged, ribbon-like fibrils are only 200–400 nm across.

3. Numerous, large mitochondria (44% of total fiber volume) have tightly packed cristae indicative of a high metabolic activity in these muscles. The cristae were observed in two configurations, normal and vesiculated.

4. The well developed sarcoplasmic reticulum occupies 19% of the total fiber volume and has a surface area of 14 μ^2/μ^3 of fiber. It completely invests the fibrils.

5. T-tube invaginations (incidence, 1 per μ^2 fiber surface) form dyads with the sarcoplasmic reticulum at the overlap regions of thick and thin filaments in each sarcomere. The thin: thick filament ratio is 3:1 as in other insect flight muscles. Thick filaments penetrate some distance into the broad Z-lines in contracted specimens.

6. Ultrastructural features of this very fast acting synchronous muscle have been compared with those of other described fast acting muscles and with other katydid flight muscles.

7. The ultrastructure of the singing flight muscles of *N. ensiger* which employs a wing frequency of 10–15 Hz during singing shows no striking differences from the singing flight muscles of *N. robustus;* the synchronous flight muscle is probably pre-adapted to a fast contraction/relaxation cycle and the greatest specialization may lie in the central nervous pacemaker and other neural characteristics.

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A STUDY OF HOMING BEHAVIOR IN THE LIMPET *SIPHONARIA ALTERNATA*[1]

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Individuals of several species of intertidal prosobranch and pulmonate limpets characteristically return to fixed locations or "homes" on rocks. These limpets move away from homes presumably to feed on algae present on rock surfaces and return to the same homes after feeding. Individual homes are usually marked by indentations or "scars" in the rock surface; the outer margins of scars often closely match the outline of the resident limpet's shell. The time of movement relative to the tidal cycle varies with the species of limpet.

Limpet homing has intrigued zoologists since the time of Aristotle (Arey and Crozier, 1921); how either prosobranch or pulmonate limpets find their homes has not been completely proven.

Four general hypotheses to explain limpet homing have been proposed. The first hypothesis is that limpets home by following clues external to their rocks (plane of polarization of light from the sky, sun or moon position, coastal landmarks, sky brightness). The second hypothesis is that animals use kinaesthetic information to navigate by a reverse-displacement or dead-reckoning system; this hypothesis is described more fully in a previous paper (S. Cook, 1969). The third hypothesis is that limpets home by using a topographic memory. The fourth hypothetical explanation is that animals follow clues which they themselves have created on rocks; such clues might include mucous trails or paths rasped in the algal cover of rocks.

Previous work has shown that the Hawaiian pulmonate limpet *Siphonaria normalis* continues to home without the use of either distant clues or reverse-displacement (S. Cook, 1969). In this paper I demonstrate that the pulmonate limpet *Siphonaria alternata* from the Florida Keys can home without using external clues, topographic memory, or reverse-displacement. I also present evidence that individual *S. alternata* home by following mucous trails.

General Materials and Methods

Field experiments

The *S. alternata* population used in all field experiments was located on small intertidal rocks on the seaward side of Ramrod Key, Florida.

Laboratory experiments

Individuals of *S. alternata* used in laboratory experiments were obtained from the Florida Keys on their original rocks. Most were collected from populations on

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Little Torch, West Summerland, and Big Pine Keys. All limpets were supplied by Mr. Stanley Becker (Tropical Atlantic Marine Specimens, P. O. Box 62, Big Pine Key, Florida).

Animals on their rocks were placed in a tidal aquarium system (Fig. 1). This system consists of two plastic aquaria connected by a discontinuous siphon. Water in the lower or reservoir tank is pumped by a Randolph peristaltic pump into a bell jar inverted above the level of the tidal aquarium. Water flows from the bell jar into the tidal aquarium through an incurrent siphon at a rate regulated by a burette attached to the siphon. When the tidal aquarium has filled to the high tide level, the discontinuous siphon begins to operate and water flows into the reservoir tank through a burette. After the tidal aquarium has emptied, an automatic timer triggers the Randolph pump to refill the bell jar and the cycle starts again. Two tidal cycles were used: (1) 6 hours of ebbing and low tide followed by 6 hours of rising and high tide per 12 hour period, and (2) 6 hours of ebbing and low tide followed by 18 hours of rising and high tide per 24 hour period. In both cycles rocks and limpets were uncovered for 2–3 hours at each low tide.

Illumination (12 hours per day) was provided by ambient fluorescent room lighting and a 100-watt incandescent bulb placed above the tidal aquarium. Aerated

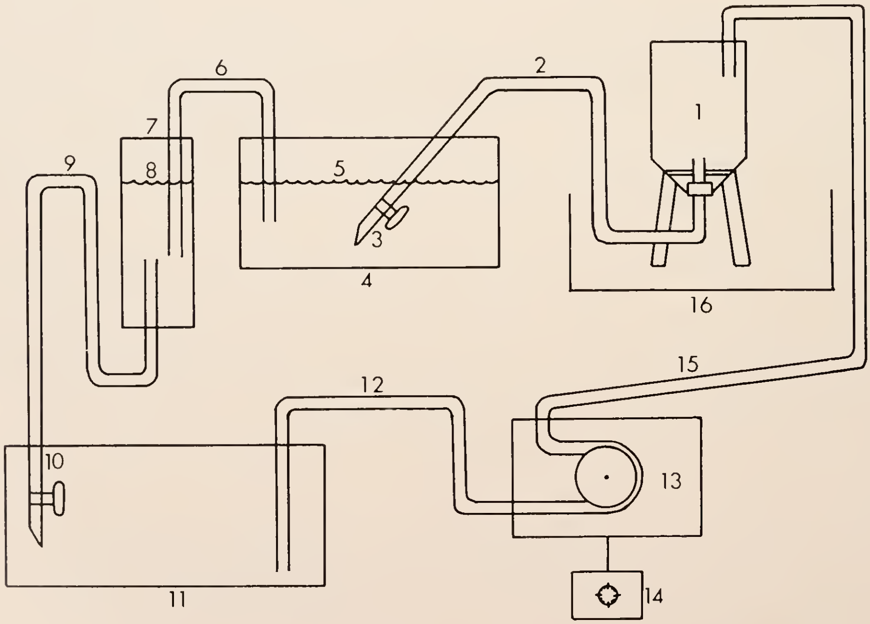

FIGURE 1. Diagram of tidal aquarium system: 1 = inverted bell jar, 2 = continuous incurrent siphon from bell jar to tidal aquarium, 3 = burette with stopcock, 4 = tidal aquarium tank, 5 = water level at which excurrent siphon is triggered, 6 = continuous siphon from tidal aquarium to plastic cylinder, 7 = plastic cylinder in which water level follows level in tidal aquarium, 8 = water level in plastic cylinder that triggers excurrent siphon, 9 = intermittant excurrent siphon from plastic cylinder to reservoir tank, 10 = burette with stopcock, 11 = reservoir tank, 12 = tubing from reservoir to Randolph pump, 13 = Randolph peristaltic pump, 14 = automatic timer, 15 = tubing from pump to bell jar, 16 = tank to catch overflow from bell jar.

"Instant Ocean" (Aquarium Systems, Inc., Wickliffe, Ohio) maintained at a salinity of 34–36‰ was used in the system; the water temperature varied from 22–25° C. The tidal aquarium was cleaned and refilled with fresh "Instant Ocean" every 6 to 14 days.

Only limpets which were active were chosen for laboratory experiments; no limpet which had been in the laboratory for more than 14 days was used.

OBSERVATIONS

Limpet behavior in the field and in the laboratory

In the field *S. alternata* moved away from homes when they were splashed by the incoming tide and when they became exposed to air on the ebbing tide. They returned to their homes by retracing their outbound paths when rocks were completely covered with water at high tide and when rocks began to dry out at low tide. Every limpet that I observed over a two week period homed. This behavior pattern is identical to that previously described for *Siphonaria japonica* (Ohgushi, 1955) and *Siphonaria normalis* (S. Cook, 1969).

Siphonaria alternata kept under artificial tidal conditions in the laboratory moved throughout the period of high tide and at low tide when rocks were damp; 71–76% of limpets moving during observation periods showed evidence of homing behavior. This percentage was considered sufficient to allow behavioral experiments in the laboratory.

Field experiments on the homing mechanism

The elimination of use of external clues in homing. Fifteen limpets were observed as they moved away from home scars; their approximate outbound paths were sketched on graph paper. When each animal began to reverse its heading at the end of its outbound trip, I rotated its rock 90° in the horizontal plane and recorded the limpet's path after rotation. I estimated the percentage of outbound path retraced by each individual.

All fifteen animals retraced 100% of outbound paths and entered their home scars. This indicates that *S. alternata* can home without using clues external to rocks.

The elimination of topographic memory as a homing mechanism. I followed outbound trips of 30 animals by drawing pencil lines along the sides of their outbound paths on rocks and sketching the approximate outlines of these paths on graph paper. After each animal had begun its return trip, it was removed from its own path and placed with its head adjacent to the freshly laid outbound path of another limpet on a topographically dissimilar rock. Each animal was replaced at a distance from the foreign scar equal to at least twice the length of its shell. In an attempt to offer each limpet only one foreign path to follow, I used only limpets in areas devoid of additional limpets. Paths of transplanted animals were followed and recorded; the percentage of foreign path followed by each displaced animal was estimated.

Twenty-two of the 30 animals retraced paths over unfamiliar topography and entered foreign scars. Four animals followed 70–80% of such paths but did not enter scars, while the remaining four did not move along the unfamiliar routes. This result indicates that use of topographic memory is not necessary for homing.

The elimination of reverse-displacement as a homing mechanism. A limpet moving along a reverse-displacement path should retrace its outbound path from its end to its beginning; the path of such an animal is shown in Figure 2b. Figure 2a represents the path of a limpet which does not follow a reverse-displacement path.

The records of 18 limpets from the preceding section were examined for evidence of reverse-displacement. In 10 of these cases, the headings of foreign paths were completely different from reverse-displacement headings. In the other 8 cases, initial headings necessary for limpets to turn onto foreign paths differed from initial headings necessary for reverse-displacement homing; after these initial differences both foreign paths and reverse-displacement paths were straight lines.

In the 10 cases in which paths were completely different, 7 animals followed foreign paths rather than reverse-displacement routes; the 3 remaining limpets did not follow the foreign trails, but rather took paths indistinguishable from reverse-displacement paths. In the 8 cases of initial difference, all animals turned to follow foreign paths. These results indicate that limpets can home without using reverse-displacement.

Laboratory experiments on homing

Ability to follow mucous trails laid on glass slides. Before use, 2″ × 3″ glass slides were cleaned with 10% (v/v) "7-X" solution, scrubbed with Alconox, and rinsed with distilled water. Grids were placed under Petri dishes filled with "Instant Ocean"; slides were placed in the Petri dishes and aligned with the grids. This allowed me to record the position of each animal throughout the experiment. Each animal was placed on a slide and allowed to lay a mucous trail; after it had done this, I removed it from the slide. The slide was rotated 90° within the dish and the water in the dish was changed. I then replaced each animal on the slide with its head next to its trail and with the long axis of its shell perpendicular to the trail. The distance from each limpet's head to either end of the trail was equal to at least twice the length of the limpet's shell. After each limpet's subsequent movements were recorded, its slide was placed in Alcian blue (0.1% (w/v) in 10% ethanol) for 1–5 minutes to stain the mucous trails. The length of each animal's

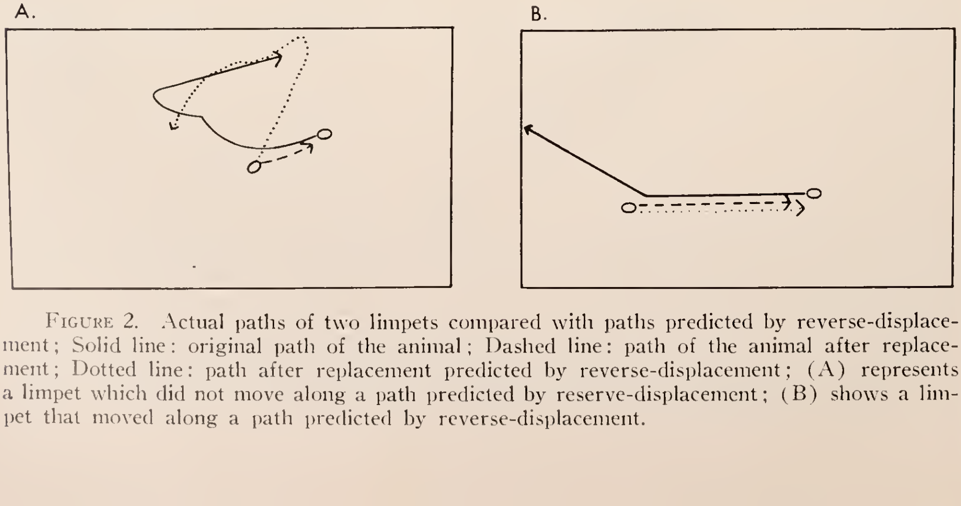

FIGURE 2. Actual paths of two limpets compared with paths predicted by reverse-displacement; Solid line: original path of the animal; Dashed line: path of the animal after replacement; Dotted line: path after replacement predicted by reverse-displacement; (A) represents a limpet which did not move along a path predicted by reserve-displacement; (B) shows a limpet that moved along a path predicted by reverse-displacement.

TABLE I

Lengths of trails available for following in laboratory experiments

Experiment	Lengths of trails available in cm		Length of trail/length of limpet shell	
	Range	Mean	Range	Mean
1	1.3–3.8	1.8	1.4–6.0	2.6
2	1.3–6.4	1.9	1.0–8.0	2.9
3 Own trails	1.0–3.8	2.0	1.6–6.0	3.2
Foreign trails	1.0–3.8	1.5	1.3–6.0	2.5

original trail and the distance that the animal's second trail overlapped the original were determined from grid records. I then computed the percentage of the original trail that each limpet retraced and compared paths followed after replacement with paths predicted by reverse-displacement.

Forty of forty-six limpets retraced 90–100% of their original trails (Fig. 3a). Lengths of trails available for following are in Table I. Thirty-three of the forty-six animals (72%) did not follow reverse-displacement paths. Before slides were stained with Alcian blue, macroscopic spots of mucous were visible at the beginnings of trails. These spots stained dark-blue; staining rendered visible, with a dissecting microscope, granules and streaks scattered along the length of each trail.

These results indicate that limpets can retrace mucous trails on glass slides which lack obvious topography without the use of clues external to the slides and reverse-displacement. Animals also can retrace their movements in the absence of paths rasped in algal cover.

Elimination of random movement and use of grid lines in trail following. It is possible that limpets in the preceding experiment may not have followed mucous trails. Rather they may have used the pattern of grid lines in some way; alternatively, when limpets lay short trails, apparent trail following may result from random movements. A simple test of these possibilities would be to remove a limpet from the end of a mucous trail, turn the slide over, and replace the animal on the clean surface at a point on the grid adjacent to its original trail as in the preceding experiment. If the animal does not retrace the path of the mucous trail, the above alternatives can be discounted.

Forty-seven limpets were tested in this way. After I removed each limpet from its trail, I selected an arbitrary point for replacement on the grid next to its trail. Slides were rotated 90° and then turned over. I changed the water in each dish, replaced each animal on the clean glass with its head at the pre-selected point, and recorded its subsequent path. I then measured the length of the original trail and the distance that the path after replacement overlapped that of the trail. From this, I computed the percentage of the path of the trail that was apparently retraced by each animal after slide inversion.

A much smaller proportion of animals (9 of 47) retraced 90–100% of their original paths (Fig. 3b) than did animals in the previous experiment (Fig. 3a). Lengths of original paths are given in Table I. The means of the distributions of percentages of trails followed in the two experiments were significantly different

($P < 0.001$, two-tailed t-test). Trail following therefore can not be explained in terms of random movement or use of grid patterns as clues; limpets apparently do detect and follow mucous trails.

Elimination of use of minor topographic features. It is possible that limpets may retrace subtle topographic features present on individual slides instead of following mucous trails. To examine this, 15 pairs of snails were allowed to lay trails on separate glass slides. Each member of a pair was then removed from its slide and replaced next to the trail of its partner. Movements before and after replacement were plotted. After each test run was finished, each animal laid another trail on a clean area of its original slide and was tested for the ability to follow it. The percentage of each kind of trail followed was calculated for each animal.

Animals followed trails of other individuals about as often as they followed their own trails (Fig. 4). Information on trail lengths is in Table I. The means of the distributions of the percentage of trails followed for the two situations were not significantly different ($P = 0.90$, two-tailed t-test). Results obtained when animals were placed on foreign trails were similar to the results of the field experiment in which limpets were placed on foreign paths ($P > 0.90$).

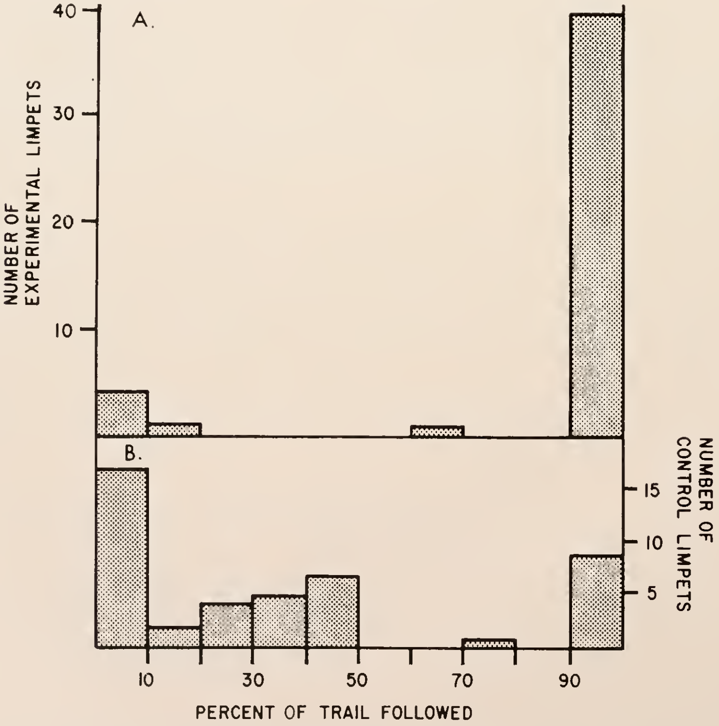

FIGURE 3. Ability of *S. alternata* to follow mucous trails; (A) experimental animals were placed next to paths of actual mucous trails; (B) control animals were placed on inverted slides in the same relative positions to trails as experimentals, except that no trails were present (see text).

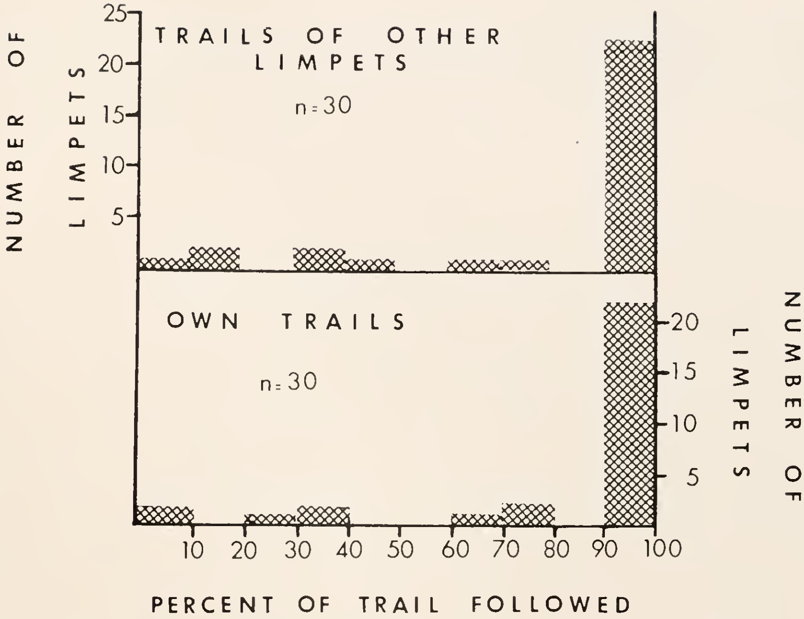

FIGURE 4. Ability of *S. alternata* to follow trails laid on slides by other individuals compared to their ability to follow their own trails.

These results indicate that animals can follow mucous trails laid by other limpets on glass slides. Memory of minor topographic irregularities that may exist on slides is therefore unnecessary for trail-following.

DISCUSSION

Results of field experiments show that *S. alternata* can home without using either distant clues, reverse-displacement or topographic memory. Laboratory experiments indicate that limpets are capable of following mucous trails (1) in the absence of major or minor features of topography, (2) in the absence of radula markings in algal cover, and (3) under conditions which should eliminate use of reverse-displacement and distant clues. The following of mucous trails laid by the limpets themselves on rocks is the simplest explanation for homing consistent with these results.

Inertial navigation is a possible means of homing that has not been considered in previous studies of limpet behavior. To home by such a mechanism an organism must have some means of measuring linear and angular acceleration as well as possess an internal clock (Barlow, 1964). The paired statocysts of pulmonate mollusks can probably detect accelerations of the magnitude of gravitational accelerations (*ca.* 980 cm/sec^2) (Charles, 1966); there is no evidence that these organs can detect the much smaller accelerations likely to be encountered in limpet homing. The fact that limpets can home to unfamiliar scars using foreign trails may indicate that such a mechanism is unnecessary for homing.

Mucous trail following is probably used by other species in the genus *Siphonaria* in homing. *Siphonaria normalis* in Hawaii can home without using external clues or reverse-displacement and must either detect information created on rocks during feeding excursions or use topographic memory (S. Cook, 1969). *Siphonaria atra, S. sipho,* and *S. japonica* all appear to home by moving along sides of previous outbound paths (Abe, 1940); this suggests that they are also following trails.

The mechanism of homing in the prosobranch limpet genus *Patella* may be similar to that used by *S. alternata.* Funke (1968) has found that several species of *Patella* follow clues that they have made on algal-covered glass plates in order to return to homes established on the plates. He concluded that limpets follow chemical clues in homing; he did not, however, consider the alternate possibilities that animals may follow radula scrapings in algal cover or may follow mucous trails by moving along textural discontinuities provided by the mucous. In a recent field study of homing in *Patella vulgata, P. depressa* and *P. aspersa,* A. Cook, Bamford, Freeman and Tiedeman (1969) have shown by rock rotation and displacement experiments that use of external clues and reverse-displacement are not necessary for homing; these results agree with Funke's (1968) explanation of homing. These authors also found that some limpets continued to home after rock surfaces around homes were scrubbed with wire brushes or treated with NaOH; they further report that chiselling trenches in rocks between limpets and homes did not prevent return. These results do not support Funke's conclusions; however, Cook *et al.* state that their experiments may not have eliminated all topographic clues or clues created by limpets.

Limpets may follow mucous trails either by following physical discontinuities of some sort created by mucous streaks or by following chemical clues. Possible chemical clues fall into 2 general categories: (1) attached, non-diffusible chemical clues such as those proposed for barnacle substrate selection during larval settling (Crisp and Meadows, 1963) and (2) chemical clues that diffuse out of trails.

In the laboratory *S. alternata* can follow trails after the trails have soaked for 48–49 hours in sea water, but do not follow trails soaked for 68–76 hours (S. Cook, 1970); experiments on the duration of effective trails on rocks in the field are lacking. It would seem likely that trails persisting for similar periods in the field would become covered with bacteria and other detritus; such accumulated debris would presumably alter the texture of any physical discontinuities characteristic of trails and might at least partially mask attached chemicals. If persistent trails exist which can be followed, this would suggest that limpets do not depend on physical discontinuities for homing clues; the probability that attached chemicals could be detected would also be decreased. Persistence of effective trails would not eliminate the use of diffusible chemicals. If such chemicals are involved, they must either (1) be present in large enough quantities so that they are detectable for 48 hours, (2) be packaged in the mucus and slowly released into the water over a period of time, or (3) not be released until limpets retrace trails. In the latter case limpets might release chemicals by the action of salivary enzymes that break down mucus; alternatively the physical action of limpet grazing might release such chemicals.

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SUMMARY

1. The pulmonate limpet *Siphonaria alternata* returns consistently to fixed "home" positions on intertidal rocks when rocks are completely covered with water at high tide and when rocks begin to dry out at low tide.

2. Limpets homed after rock rotation, were able to follow paths made by other limpets on foreign rocks, and did not follow paths characteristic of reverse-displacement. These results eliminate use of external clues, topographic memory, and reverse-displacement and indicate that limpets home by using clues that they have previously created on rocks.

3. Limpets can follow mucous trails that they have previously laid on clean glass slides in the laboratory. This result indicates that limpets can follow mucous trails without using paths made by radula marks in algal cover. This behavior does not result from random movements.

4. The above results support the hypothesis that homing *S. alternata* retrace mucous trails that they have laid previously.

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THE EFFECTS OF FOOD DEPRIVATION AND SALINITY CHANGES ON REPRODUCTIVE FUNCTION IN THE ESTUARINE GOBIID FISH, *GILLICHTHYS MIRABILIS*

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Photoperiod and temperature are frequently considered the most important proximate factors regulating teleost reproductive cycles (de Vlaming, 1972); yet few investigators have examined the influence of other environmental factors, such as changes in food availability, salinity, and oxygen concentration. A previous investigation (de Vlaming, 1971) showed that the spawning period in the estuarine gobiid fish, *Gillichthys mirabilis*, is protracted, extending from December to June. Gonadal regression occurs rather abruptly in July; the gonads remain regressed during August and September. Gonadal recrudescence begins in late September, reaching completion by early December. Evidence presented by de Vlaming (in preparation) indicates that the increasing temperatures of summer may be responsible for terminating reproduction in the Alviso population of *G. mirabilis*. Carpelan (1957), however, in a study of the hydrobiology of the Alviso ponds, showed that there is a decrease in productivity, increase in salinity, and decrease in oxygen concentration in this habitat during the summer.

While there is a general awareness of a nutritional influence on fertility and fecundity, little information is available concerning specific nutritional effects on the gonads of fishes (Fontaine and Fontaine, 1962). Bagenal (1967), Nikolsky (1963), and Woodhead (1960) reviewed the literature on fish fecundity, and indicated that fecundity generally decreases with decreasing food availability. With regard to salinity, Kinne (1964) stated that this factor usually affects reproduction of marine and brackish animals less obviously than temperature. Kinne also suggested that salinity changes are seldom of importance in timing annual breeding cycles. The effects of salinity on fish reproduction, however, have been investigated in only a few species; nonetheless, in the Baltic Sea sterility or reduced reproductive potential due to low salinity has been reported for several pleuronectid fishes (Marx and Henschel, 1939).

The seasonal timing of gonadal regression in *G. mirabilis* suggests that decreasing food availability or increasing salinity could be implicated. Accordingly experiments were designed to assess these possibilities.

MATERIALS AND METHODS

To determine seasonal variation in fattening, monthly samples of *Gillichthys* from the Alviso ponds of San Francisco Bay (37° 27′ N) in California were obtained by trapping with modified minnow traps. Samples were usually taken near the middle of each month. Collections were begun in December 1969 and continued until October 1970. Formalin (8%) preserved specimens of *Gillichthys* were obtained from the Scammons Lagoon population (27° 48′ N) in Baja California between April and October 1970. The fish from the Alviso population were killed on the day of collection using a saturated solution of chlorotone. Only males longer than 125 mm and females longer than 120 mm were used in these studies to keep animal size as uniform as possible.

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Samples of fish for experimental purposes were captured in the Alviso habitat at different times during the year and thus in different phases of gametonenesis. Several fish from each sample were sacrificed and the gonads examined at the time of capture; these fish, the initial controls, served as a reference for the experiments that followed.

Gonadal weights are expressed in absolute terms since it has been shown (de Vlaming, 1971) that gonadal weight is independent of body weight (and length) in the size of fish utilized. Gonads were fixed in Bouin's, Zenker's, alcoholic Bouin's, or Zenker-formol solutions and embedded in paraffin for histological examination. Tissues were sectioned at 5 μ–8 μ and stained with Delafield's, Harris', or Heidenhain's haematoxylin and counterstained with eosin or light green. Some

TABLE I

Criteria used in evaluating gametogenetic activity in G. mirabilis

Stage	Histological characteristics of testes
0	"Regressing testis." Seminiferous lobules characterized by large numbers of pyknotic nests of degenerating cells (spermatozoa, spermatids, and spermatocytes); phagocytes observed free within the lobules.
1	"Quiescent testis." Seminiferous lobules small in diameter. Germinal epithelium consists of only few residual spermatozoa, and the sperm duct is collapsed.
2	"Mitotic phase." Same as Stage 1, with the exception that mitotic figures are observed in the spermatogonia.
3	"Meiotic phase or active spermatogenesis." Testicular lobules larger than in Stages 1 and 2; germinal epithelium consists of spermatogonia, spermatocytes, and spermatids.
4	"Pre-spawning testis." Seminiferous lobules large and distended with sperm. Germinal epithelium consists of relatively few spermatogonia.
5	"Post-spawning testis." Seminiferous lobules small and contain relatively few sperm; sperm duct expanded and containing residual sperm.
	Histological characteristics of ovaries
I	"Regressing ovary." Atretic follicles predominate in the ovary. Only non-yolky oocytes and oogonia present.
II	"Quiescent phase or phase of oogonial proliferation." Ovary characterized by non-yolky oocytes with a basophilic cytoplasm, and a diameter of less than 75 μ. Granulosa not fully organized around the developing oocytes.
III	"Phase of active vitellogenesis." Ovary characterized by developing yolky oocytes whose diameter is between 75 μ and 640 μ. Granulosa fully organized around the oocytes.
IV	"Pre-spawning condition." Ovary characterized by oocytes whose diameter is in excess of 640 μ. Yolk vesicles abundant.
V	"Post-spawning condition." The ovary is wine-red in color; the tunica albuginea thick, highly vascularized, and folded. Post-ovulatory follicles predominate in the ovary. The stroma of the ovary appears disorganized, yet highly vascularized.

testes were also stained with Sudan Black B or by the PAS technique (Humason, 1962). Spermatogenesis and oogenesis were divided into six and five recognizable phases (Table I), respectively, to facilitate quantitative evaluation of gametogenetic activity. These phases have been previously described by de Vlaming (1971).

Stage III of this arbitrary classification of ovaries could be divided into several phases of vitellogenesis; however, a single category is used here to denote a stage of active vitellogenesis. Egg diameter is not used since oocyte development is not synchronous in this species (de Vlaming, 1971); oocytes of varying diameter, and different phases of vitellogenesis characterize ovaries undergoing active gametogenesis.

In salinity experiments, increased osmotic concentrations were achieved by adding Seven Seas Marine Mix (Utility Chemical Co.) to natural sea water; osmotic concentrations were determined by using an American Optical refractometer (Model 10402). Concentrations were maintained at a constant level in each experiment. The fish in these experiments were provided with a varied diet consisting of brine shrimp, chopped fish, boiled egg-white, beef kidney and liver. In the starvation experiments, the weight-length ratio of each fish was determined by dividing body weight (less the weight of the gonads) by the standard length. The hepatosomic index was determined by dividing the liver weight by the body weight (less the weight of the gonads). These two indices reflect the robustness (and hopefully nutritional state or energy reserves) of the fish.

Experimental fish were maintained in 56- or 132-liter tanks. Recirculating filtered water was used in all of these experiments. Tanks were housed in constant temperature rooms (± 1.5° C) and water pH maintained between 8.0 and 9.5 (which is consistent with the Alviso habitat).

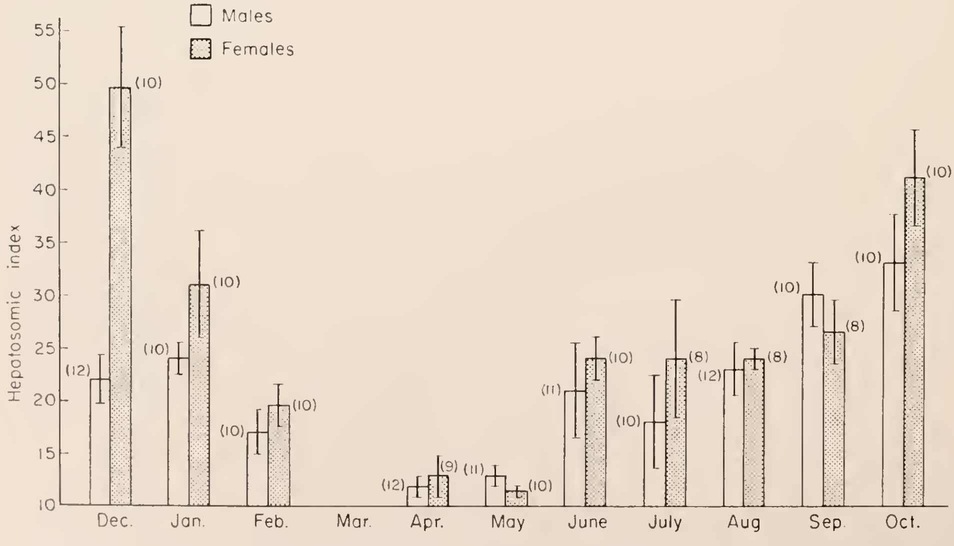

FIGURE 1. Seasonal variation (Dec. 1969–Oct. 1970) in hepatosomic index of *G. mirabilis* from Alviso population. Histograms (shaded = females; open = males) represent means; means are bracketed by one standard error. Sample size is shown in parentheses; hepatosomic index = (Liver wt/Body wt less gonadal wt), × 100.

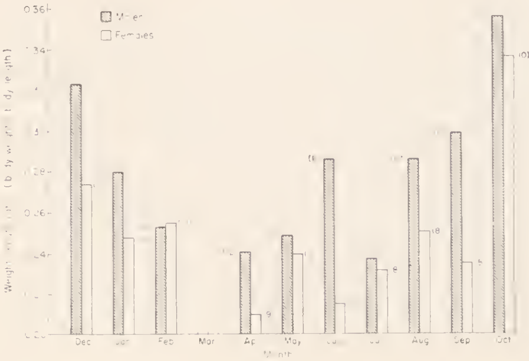

FIGURE 2. Seasonal variation (Dec. 1969–Oct. 1970) in weight-length ratio of *G. mirabilis* from Alviso population. Histograms (shaded = males; open = females) represent means; standard error less than 0.005 in all groups. Sample size is shown in parentheses; weight-length ratio = Body wt (g) less gonadal wt/Body length (mm).

Statistical comparisons of gonadal weights between experimental groups were made by using the Mann-Whitney U test (Siegel, 1956). This nonparametric test is suitable for small sample sizes and can be used to determine whether two independent groups have been drawn from the same population.

Individual experiments were conducted to determine the effects of inanition on fish in a phase of active gametogenesis, on the rate of testicular regression at a high temperature and on gonadal recrudescence. Other experiments were initiated to examine the influence of high salinity on gonadal regression and recrudescence in *Gillichthys*.

RESULTS

Nutrition

Seasonal variation in hepatosomatic index and weight-length ratio. Seasonal changes in weight-length ratios and hepatosomic indices are illustrated in Figures 1, 2, 3 and 4. In the Alviso and Scammons Lagoon populations both the weight-length ratio and hepatosomic index begin to increase towards the end of the spawn-

TABLE II

Effect of 3-week starvation on gonadal weight in G. mirabilis *(16° C)*

Treatment	Gonadal weight ($\bar{x}$ ± S.E.)		Hepatosomic index ($\bar{x}$ ± S.E.)	
	Testes (n) (mg)	Ovaries (n) (mg)	Male	Female
Initial controls (from natural population)	79.1 ± 9.5 (10)	474 ± 23 (8)	16.9 ± 2.3	18.6 ± 2.8
Fed	85.4 ± 6.2 (10)	506 ± 17 (8)	28.4 ± 5.0*	30.0 ± 3.5*
Starved	43.5 ± 4.5* (10)	245 ± 9* (8)	9.8 ± 1.3*	10.4 ± 0.9*

* Significantly different ($P < 0.01$) from initial controls.

ing season (May or June). These indices continued to increase as gonadal recrudescence was occurring (until October in the Alviso population and August in the Scammons Lagoon population). With the onset of spawning (December in the Alviso population and September in the Scammons Lagoon population) both the weight-length ratio and hepatosomic index began to decrease, and continued to decrease through the spawning season in the Alviso population. These data indicate that there is a seasonal variation in the robustness (and perhaps energy reserves) of *Gillichthys* which is correlated with the reproductive cycle.

The effects of inanition in January. To determine whether food shortage could initiate gonadal regression an experiment was begun in January when the testes of fish were in active spermatogenesis, the pre-spawning, or post-spawning condition (Stages 3, 4, and 5); the ovaries of fish in this sample were in phases of active vitellogenesis (Stage III). One group of fish was fed every other day, whereas another group received no food; both groups were placed at 16° C and sacrificed after 23 days (Table II). In healthy fish, gonads remain active at this temperature (de Vlaming, in preparation).

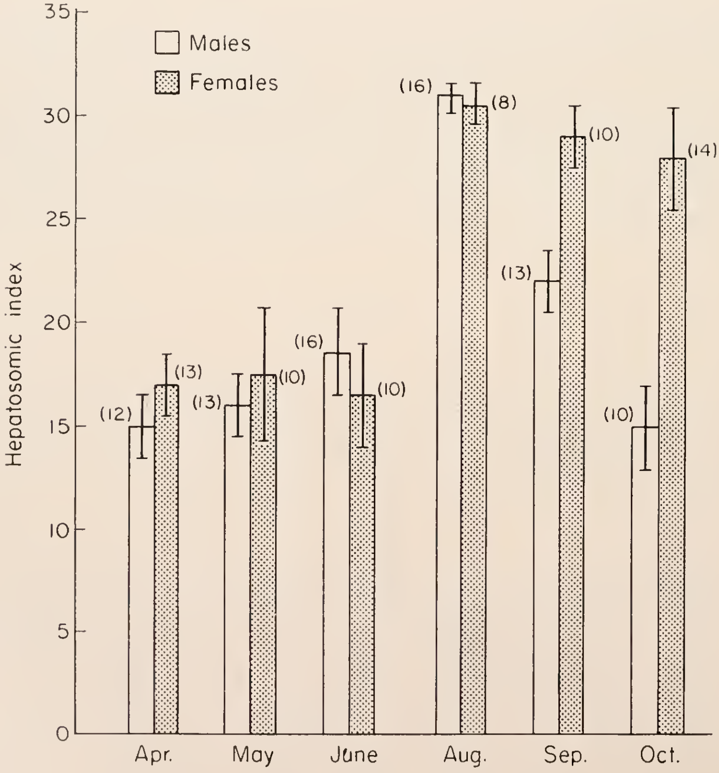

FIGURE 3. Seasonal variation (April–Oct. 1970) in hepatosomic index of *G. mirabilis* from Scammons Lagoon population. Histograms (shaded = females; open = males) represent means; means are bracketed by one standard error. Sample size is shown in parentheses; hepatosomic index = (Liver wt/Body wt less gonadal wt), × 100.

TABLE III

Rate of testicular regression in starved and fed G. mirabilis *at 27° C*

	Testicular weight (mg) $\bar{x} \pm$ S.E. (n)	Hepatosomic index ($\bar{x} \pm$ S.E.)	Body wt/body length** ($\bar{x}$)
Initial controls (from natural population)	89.2 ± 7.1 (10)	13.33 ± 1.04	0.259
Fed	67.8 ± 6.7* (10)	18.88 ± 2.13*	0.270*
Starved	55.3 ± 7.5* (9)	6.26 ± 0.88*	0.245*

* Significantly different ($P < 0.01$) from the initial controls.
** Standard error < 0.001 in all groups.

Inanition for 23 days caused significant decreases ($P < 0.01$) in both ovarian and testicular weights compared to the initial controls and fed fish. The testes of the starved fish were regressing or in the quiescent phase (Stage 0 or 1); ovaries of fish in this group were also regressing (Stage I). In contrast, the gonads of the fed fish remained in the initial condition. The significant decrease ($P < 0.01$) in the hepatosomic index of the starved fish indicates that energy reserves were taxed by the lack of food.

Rate of testicular regression in starved and fed fish at 27° C. A second experiment was undertaken in May to ascertain the effects of nutrition on the rate of heat-induced testicular regression; testes of the initial controls collected from the natural population were in active spermatogenesis or the pre-spawning condition (Stages 3 or 4). One group of fish was fed every day *ad libitum*, whereas another group received no food; both groups were placed at 27° C (which causes gonadal regression in fed fish, de Vlaming, in preparation) and sacrificed after ten days (Table III).

Testicular weights of fish in both the starved and fed groups were significantly lower ($P < 0.01$) than those of the initial controls, but were not significantly different from one another; the testes of all fish were regressing (Stage 0). The hepatosomic index and the weight-length ratio of the starved fish were significantly lower ($P < 0.01$) than those of the initial controls, but those of the fed fish had increased significantly ($P < 0.05$). Thus, in this short-term experiment the rate of testicular regression was not accelerated by starvation.

TABLE IV

Effect of 40-day starvation on gonadal recrudescence in G. mirabilis *(20° C)*

Treatment	Gonadal weight ($\bar{x} \pm$ S.E.)		Body wt/body length** ($\bar{x}$)	
	Testes (n) (mg)	Ovaries (n) (mg)	Male	Female
Initial controls (from natural population)	27.3 ± 1.3 (8)	114 ± 42 (8)	0.294	0.247
Fed	58.0 ± 3.7* (6)	408 ± 48* (6)	0.296	0.251
Starved	43.6 ± 4.1* (6)	351 ± 32* (6)	0.176*	0.181*

* Significantly different ($P < 0.01$) from initial controls.
** Standard error < 0.001 in all groups.

Effect of 40-day starvation on gonadal recrudescence (20° C). To determine whether diet limitations could inhibit the initiation of gonadal recrudescence the effect of starvation was examined in July when the testes and ovaries of the initial controls were regressing (Stage 0 and I). Controls were fed *ad libitum* every other day. Fish were placed at 20° C and sacrificed after 40 days (Table IV).

Testicular and ovarian weights of both experimental groups increased significantly ($P < 0.01$); active spermatogenesis (Stage 3) and vitellogenesis (Stage III) had been initiated in the gonads of all fish. Although the initiation of recrudescence was not blocked by inanition, the rate of recrudescence may have been reduced since the testicular and ovarian weights in the fed group were significantly higher ($P < 0.05$) than those of the starved group. The weight-length ratio of

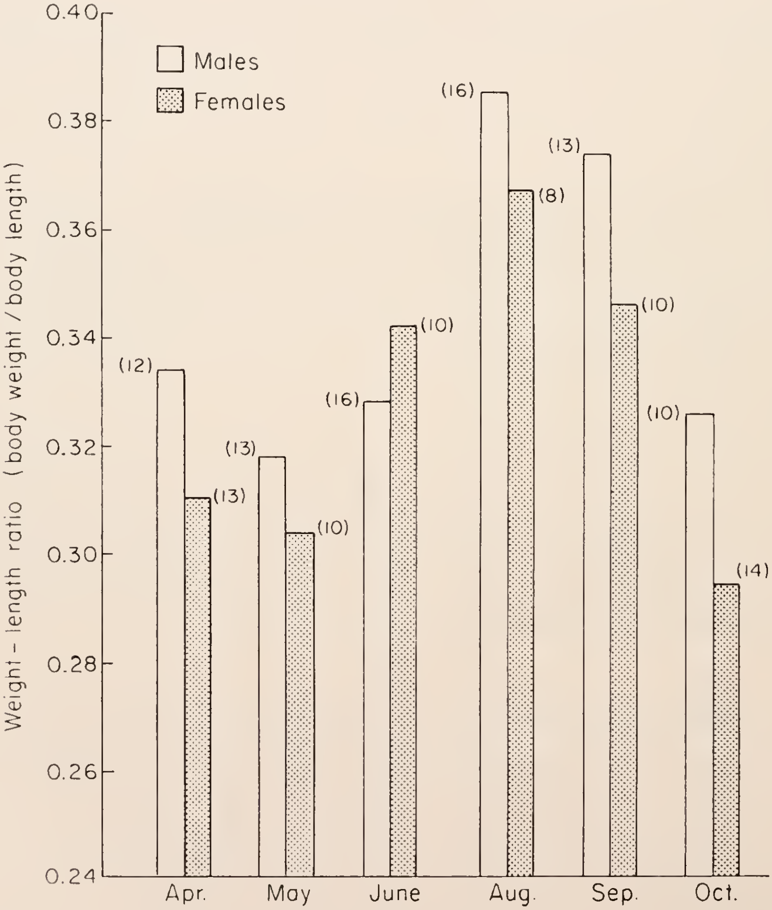

FIGURE 4. Season variation (April–Oct. 1970) in weight-length ratio of *G. mirabilis* from Scammons Lagoon population. Histograms (shaded = females; open = males) represent means; standard error less than 0.005 in all groups. Sample size is shown in parentheses; weight-length ratio = Body wt (g) less gonadal wt/Body length (mm).

TABLE V

Effect of 80-day starvation on gonadal weight in G. mirabilis *(16° C)*

Treatment	Gonadal weight ($\bar{x}$ ± S.E.)		Hepatosomic index ($\bar{x}$ ± S.E.)		Body wt/body length** ($\bar{x}$)	
	Testes (n) (mg)	Ovaries (n) (mg)	Male	Female	Male	Female
Initial controls (from natural population)	32.2 ± 2.2 (12)	125 ± 6 (8)	19.4 ± 7.7	26.9 ± 11.3	0.265	0.248
Fed	54.6 ± 4.1* (8)	342 ± 21* (8)	37.2 ± 6.4*	41.6 ± 5.8*	0.354*	0.329*
Starved	49.8 ± 3.9* (12)	309 ± 14* (8)	8.1 ± 1.1*	12.3 ± 0.5*	0.208*	0.184*

* Significantly different ($P < 0.01$) from initial controls.
** Standard error < 0.001 in all groups.

both male and female starved fish was significantly less ($P < 0.01$) than those of the initial controls, suggesting that starvation did cause a decrease in body weight.

Effect of 80-day starvation on gonadal recrudescence (16° C). The effects of longer-term starvation on gonadal recrudescence at a moderate temperature were again examined in July; the testes and ovaries of all of the initial controls were regressing (Stage 0 and I). The conditions employed were the same as in the previous experiment, except fish were placed at 16° C and sacrificed after 80 days (Table V).

Testicular and ovarian weights of both experimental groups increased significantly ($P < 0.01$); active spermatogenesis (Stage 3) and vitellogenesis (Stage III) were initiated in the gonads of all fish. Gonadal weights of the starved and fed fish were not significantly different. The hepatosomic index and weight-length ratio of both male and female starved fish were significantly less ($P < 0.01$) than those of the initial controls.

Salinity

Effects of high salinity on gonodal regression. To determine whether high salinity could induce gonadal regression, an experiment was begun in October in which the testes of the initial controls were in active spermatogenesis (Stage 3) and ovaries were in phases of vitellogenesis (Stage III). One group of fish was

TABLE VI

The effect of 20-day high salinity treatment on gonadal regression in G. mirabilis *(20° C)*

Treatment	Gonadal weight ($\bar{x}$ ± S.E.)	
	Testes (n) (mg)	Ovaries (n) (mg)
Initial controls (from natural population)	68.4 ± 10.3 (10)	2394 ± 404 (10)
35 parts/thousand	90.1 ± 5.2* (9)	2458 ± 217 (6)
70 parts/thousand	75.7 ± 9.1 (10)	2263 ± 195 (5)

* Significantly greater ($P < 0.01$) than initial controls.

Table VII

The effect of 70-day high salinity treatment on gonadal recrudescence in G. mirabilis *(20° C)*

Treatment	Gonadal weight ($\bar{x}$ ± S.E.) Testes (n) (mg)	Ovaries (n) (mg)
Initial controls (from natural population)	23.9 ± 1.6 (8)	158 ± 24 (8)
35 parts/thousand	109.5 ± 8.7* (6)	1776 ± 152* (6)
70 parts/thousand	56.1 ± 3.6* (6)	453 ± 17* (5)

* Significantly greater ($P < 0.01$) than initial controls.

exposed for 20 days to a salinity of 35 parts per thousand (ppt) and another group to 70 ppt; both groups were at 20° C (Table VI).

Ovarian condition in both experimental groups remained at the initial level. Testicular weights of fish at the lower salinity increased significantly ($P < 0.01$), and were also significantly greater ($P < 0.05$) than those of fish at the high salinity. Histological examination, however, revealed that the testes of fish in both groups were in Stages 3 or 4. These data suggest that high salinity may reduce the rate of gametogenesis, but nonetheless, gonadal regression is not stimulated.

Effects of high salinity on gonadal recrudescence. To determine whether high salinity would prevent gonadal recrudescence in *Gillichthys* an experiment was begun in September 1967 in which the testes and ovaries of the initial controls were in the quiescent phase (Stage 1 and II). One group of fish was exposed for 70 days to a salinity of 35 ppt and another group to 75 ppt both at a temperature of 20° C (Table VII).

Testicular and ovarian weights in both groups increased significantly ($P < 0.01$), but those of fish in the low salinity group were significantly greater ($P < 0.01$) than testicular and ovarian weights in the high salinity group. The testes of all fish in the low salinity group were in the pre-spawning condition (Stage 4), whereas those of fish at 75 ppt were in Stage 3. The ovaries of all fish at the high salinity were in the early phases of vitellogenesis (Stage III), whereas those of the fish at 35 ppt were in later phases of vitellogenesis. These data indicate that high salinity does not block the initiation of gonadal recrudescence, but does reduce the rate of gametogenesis.

Discussion

The effects of starvation on gametogenesis in *G. mirabilis* vary as a function of season. This variability in the response to inanition may depend on the energy reserves of the initial controls or susceptibility of the gonadotropin-producing system. The data presented here indicate that starvation can induce gonadal regression in a relatively short period in *Gillichthys* in phases of active gametogenesis. In sexually maturing *Salmo gairdneri* starvation also reduces the number of eggs brought to maturity by causing follicular atresia (Scott, 1962). Clemens and Reed (1967) also showed that spermatogenesis in *Carassius auratus* is terminated by diet limitations at any time of the year. An increase in feeding has been shown to hasten the onset of sexual maturity by a year in *Pleuronectes limanda* (Gross, 1949), *Clupea harengus* (Cushing and Burd, 1956) and *Salvelinus alpinus* (Runnström, 1951), whereas poor feeding delayed maturity in *Perca fluviatilis* (McCay, Dilley and Crowell, 1928–9; Alm, 1954) and *Salmo trutta* (Bagenal, 1969).

Perhaps then, decreasing food availability and the increasing temperatures of summer act synergistically to cause gonadal regression in the Alviso population of *Gillichthys*. Temperature does indeed have a pronounced effect on fish when food availability is low. For example, Phillips, Livingston and Dumas (1960) showed that during starvation weight loss is increased in brook trout by approximately 10% for each 1° C rise in water temperature. High temperature alone, however, can cause gonadal regression in *Gillichthys* because in all of the experiments conducted by de Vlaming (in preparation) thermally regressed fish were well fed (weight-length ratios and hepatosomic indices did not decrease). In addition, data presented here imply that the rate of gonadal regression at high temperatures is not increased by starvation. Furthermore, the complete starvation used in these experiments probably represents a more severe nutritional stress than is actually encountered by fish in nature.

Although metabolic rate in fish increases with temperature, food consumption may not increase sufficiently to maintain fat reserves and body weight. Creach and Serfaty (1965) indicated that the gonads and muscles are the principle source of free amino acids for metabolism when *Cyprinus carpio* is subjected to starvation. In addition, Kinne (1960) reported that the efficiency of food conversion in *Cyprinodon macularis* is maximal at lower temperatures and salinities, declining at higher temperatures and salinities. Paloheimo and Dickie (1966), nonetheless, indicated that increases in temperature increase the rate of energy turnover, but do not otherwise alter the basic pattern of distribution and use of energy within the body of fishes. Moreover, the weight-length ratio and hepatosomic index in *Gillichthys* begins to increase as gonadal regression occurs so it seems unlikely that energy shortage causes gonadal regression. Mann (1965) has also reported that, in several species of fish, withholding food reduces metabolic rate 50% within seven days. Possibly, however, food restriction leads to gonadal regression indirectly by causing stress and subsequent changes in the endocrine system.

The data presented here indicate that starvation will not block the initiation of gonadal recrudescence in *Gillichthys*. In contrast, Wilkins (1967) noted that starved *Clupea harengus* failed to undergo gonadal recrudescence. Assenmacher, Tixier-Vidal and Astier (1965) reported that starvation failed to prevent gonadal recrudescence in ducks, but fasting did induce involution of developing gonads. Sluiter, van Oordt and Grasvelt (1950) also showed that the effect of inanition on spermatogenesis in a frog, *Rana temporaria*, depends on the time of year; when fat bodies are large starvation has little effect, but when they are small inanition inhibits spermatogenesis. In this study, however, experiments on recrudescence were begun in July when the length-weight ratio and hepatosomic index of fish were relatively low (lower than in January when starvation brought about gonadal regression). These two indices should be indicative of the nutritional state of fish since Woodhead (1960) showed that the main source of fat and protein used during gonadal maturation comes from the liver and muscles. The variation in response to starvation in *Gillichthys* could be due to seasonal shifts in metabolism; such shifts are common in fish (Wells, 1935; Wohlschlag and Juliano, 1959; Beamish, 1964;

Roberts, 1964). However, temperatures are higher in early autumn (when starvation failed to prevent the initiation of recrudescence) and metabolism should be higher, than in January (when starvation caused testicular regression). In addition, Barlow (1961) reported that oxygen consumption remains the same throughout the year in *Gillichthys* from the Alviso population (measured at the same temperature).

If the weight-length ratio and hepatosomic index can be taken as an indication of fattening (or energy accumulation), then this process occurs during the autumn concomitant with gonadal recrudescence. Likewise, as spawning begins, these two indices begin to decline and continue to decrease through the spawning season, suggesting a depletion of energy reserves. Healey (1971) has also noted that changes in body weight in *Gobius minutus* are closely correlated with reproductive cycling. The decline in hepatosomic index during the spawning season (when estrogens should be high) is surprising since Kobayski (1953), Egami (1955) and Oguro (1956) showed that estrogens increase liver weight in fish. Perhaps in this species the prolonged spawning season places a burden on energy reserves, and energy expenditure overrides the estrogen effects on the liver. Indeed, a decrease in energy reserves during the spawning season might be expected since sex hormones increase the rate of oxygen consumption in fish (Raffy and Fontaine, 1930; Stanley and Tescher, 1931; Mann, 1939; Hasler and Meyer, 1942). According to Wilkins (1967), *Clupea harengus* exhibit their lowest fat content after the spawning season, and Lofts, Pickford and Atz (1968) indicated that *Fundulus heteroclitus* with regressed gonads have large livers. In *Gillichthys*, growth is fastest in the hot summer months (Walker, 1961) when the gonads are regressed.

Although variation in food availability is apparently not the proximate factor regulating reproductive cycling in *Gillichthys*, it well could be the ultimate control factor. Low food availability would probably not favor the survival of fry, and indeed, Ivlev (1961) found that younger fish have a shorter survival time than older fish in starvation experiments.

Studies with other euryhaline species indicate that salinity can influence reproduction. *Mugil cephalus* and *M. capito* cannot reproduce in freshwater (but spend part of each year there), and oocytes remain in the previtellogenic stage (Abraham, Blanc and Yashouv, 1966; Abraham, Yashouv and Blanc, 1967). The gonadotropin content of the pituitary of these *Mugil* species held in freshwater is considerably lower than that of pituitaries from sea-water fish (Blanc and Abraham, 1968), and the area of the pituitary containing the gonadotrophic cells is reduced in size in fresh-water specimens (Blanc-Livni and Abraham, 1970). Low salinities normally experienced by *Pleuronectes flesus* also block vitellogenesis (Solemdal, 1967).

Salinity in the Alviso ponds reaches a maximum in summer (August and September) when the gonads of *Gillichthys* are regressed; during this time salinity seldom exceeds 55 ppt in the ponds in which this species occurs (Carpelan, 1957). The experiments reported here (using salinities of 70 and 75 ppt) indicate that high salinity is not responsible for gonadal regression, nor will it prevent the initiation of gonadal recrudescence. Weisel (1948) also stated that the spermatozoa of *Gillichthys* are active in salinities ranging from 17 to 200% sea water. In combination with high temperatures, high salinity may induce gonadal regression, but changes in salinity alone cannot be considered a proximate factor in regulating reproductive cycling.

High salinity could act as an ultimate control factor with regard to reproductive cycling by influencing larval survival. This is doubtful, however, since Blaxter (1969), in a review of the literature, indicated that the salinity tolerance of larvae and eggs of marine fish is surprisingly wide.

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SUMMARY

1. Investigations were conducted to examine whether decreasing food availability or increasing salinity might be implicated in termination of the breeding season in the estuarine gobiid fish, *Gillichthys mirabilis*.

2. Seasonal variation in weight-length ratio and hepatosomic index were studied in two populations of this species. These two indices, taken as an indication of fattening (or energy accumulation), seem to be correlated with the reproductive cycle. Both indices begin to increase as the gonads regress, and continue increasing concomitant with recrudescence; they decline steadily through the spawning season.

3. The effects of starvation on gametogenesis in this fish vary with season. Starvation can induce gonadal regression in a relatively short period in fish in phases of active gametogenesis, but does not block the initiation of gonadal recrudescence.

4. The rate of gonadal regression at high temperatures is not accelerated by starvation, suggesting that temperature can act independently to cause gonadal involution.

5. High salinity does not cause gonadal regression nor prevent recrudescence in *Gillichthys*.

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BEHAVIORAL SPECIFICITY AND THE INDUCTION OF HOST RECOGNITION IN A SYMBIOTIC POLYCHAETE[1]

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Chemically mediated behavioral phenomena are common throughout the animal kingdom and have been the subjects of numerous recent reviews (Davenport, 1966; Butler, 1967; Blum, 1969; Lenhoff, 1968; Schoonhoven, 1968; Regnier and Law, 1968; Gleason and Reynierse, 1969). These investigations have clearly demonstrated the importance of specific chemical information in the mediation of countless inter- and intraspecific relationships. Indeed, chemical communication appears to be the paramount mode of communication in most groups of animals (Wilson, 1970).

Several kinds of chemically mediated phenomena are known to be susceptible to modification through some kind of conditioning process. Thorpe and Jones (1937) introduced the concept of "olfactory conditioning" to describe the effect of exposure to an abnormal host on the subsequent host selection behavior of an insect parasite. Similarly, Cushing (1941) invoked such a mechanism to explain substrate preference for oviposition by *Drosophila*. Numerous additional accounts of the role of previous experience in various chemically mediated behavioral phenomena appear in the literature on insect behavior (Dethier, 1970).

Selection of the particular stream leading to their birthplace by anadromous fishes (Hasler and Wisby, 1951), food preference in various vertebrates (Ivlev, 1961; Burghardt, 1966; Burghardt and Hess, 1966), and prey selection by three predatory marine invertebrates, the Pacific starfish *Pisaster* (Landenberger, 1968) and the gastropods *Urosalpinx* (Wood, 1968) and *Acanthina* (Murdoch, 1969), are additional chemically mediated phenomena affected by conditioning. The concepts "chemical imprinting" (Burghardt, 1966) and "ingestive conditioning" (Wood, 1968) have been introduced into the literature as a result of these kinds of investigations. It appears that these chemical conditioning phenomena are widespread throughout the animal phyla.

It has been clearly demonstrated (see Davenport, 1966) that several groups of symbiotic polychaetes are capable of recognizing and responding to some chemical signal emanating from their hosts. Among these polychaetes the polynoid genus *Arctonoe* presents an interesting complex of host-symbiont associations that are amenable to comparative experimental analyses. One species in this genus, *Arctonoe pulchra*, is of particular interest in that this worm is associated with at least nine species representing five classes in three phyla (Pettibone, 1953; Dimock, 1970). Previous work with this species has been limited to a demonstration of a chemotactic response by *A. pulchra* to one of its hosts, the sea cucumber *Stichopus californicus* (Davenport, 1950; Davenport and Hickok, 1951). In the investigations reported here populations of this worm from four additional host species have been examined with regard to the occurrence of chemically mediated host recognition behavior, the specificity of these responses, and the effect of the past experience of individual worms on their subsequent host recognition behavior.

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METHODS

Several designs of olfactometers were tested for use as an assay for the host recognition behavior of *Arctonoe pulchra*. It soon became obvious that any apparatus employing multiple worms simultaneously was of no use with this species as these worms are particularly aggressive towards each other and cannot be confined with one another. The design finally settled upon was a modification of the Y-maze choice apparatus described by Davenport (1950). This device, constructed of $\frac{1}{2}$ inch i.d. Lucite tubing, consisted of a 10 cm stem connected to two 8 cm arms of the Y 60° apart. The arms terminated at a 45° elbow. The stem was fitted with a partial partition and drain post at its free end. The whole device was mounted on a sheet of $\frac{1}{4}''$ Lucite provided with 3 brass bolt legs for leveling. Thus, test solutions could be introduced at the arms of the Y, the device leveled, and the solutions allowed to drain slowly from the stem, a procedure which resulted in the apparatus being maintained with a slowly flowing solution which filled the tube to approximately $\frac{1}{3}$ to $\frac{1}{2}$ full.

There are several inherent limitations and difficulties associated with a choice apparatus of this design. Since test organisms must be employed one at a time in such a device, considerable time may be spent in amassing enough data for statistical analysis. Depending upon the arrangement of the organism's chemoreceptors which mediate the response under investigation, it is possible that the subject could be stimulated by effluents from only one of the two arms of the maze. It is also quite possible that an individual organism may exhibit a preference for either the left or the right arm of the Y-maze (Putnam, 1962), a preference that may either be genetic or simply a response to some trails or traces from animals in previous trials. Occasionally, an animal may make an over-shoot mistake at the junction of the two arms as a result of the sudden confrontation at the sharp demarcation between two alternate currents. The most important limitation, however, is the lack of an opportunity for the analysis of the full behavior of the animal evoked by chemical signals, since the response is limited to a directed response canalized to a simple "choice" (Gage, 1966).

These limitations have been acknowledged in this study and efforts made to minimize their effect on the interpretation of the results. Individual worms have not been used repeatedly in single experiments. The presentation of test solutions into the arms of the Y-maze was randomized. The tubes were thoroughly rinsed in clean sea water between individual tests. Finally, the observed responses are recognized as being only part of a behavior pattern which might effect contact between a symbiont and its host and are not considered to be the sole basis of host recognition by these organisms.

Test solutions (hereafter referred to as test or host effluents) were prepared by allowing test organisms (enough total mass to displace 500 ml) to stand in 3000 ml of aerated filtered sea water for 20–24 hours prior to testing. Any irregularity in the production of an attractant by a test organism was compensated for by using this lengthy time period and typically 2 or 3 test organisms per 3000 ml. Effluent thus prepared was then siphoned into the arms of the Y-maze. Since preliminary experiments suggested that there was little effect of flow rate on the worms' behavior, the flows in the two arms of the Y were arbitrarily balanced at 0.5 ml/arm/7–10 sec with polyethylene buret tips. Temperatures were regulated by water baths (11–14° C, Friday Harbor; 15–19° C, Santa Barbara).

The organisms employed in these experiments were collected by hand while diving or were dredged. In Santa Barbara, California, the sea cucumber *Stichopus parvimensis* and the limpet *Megathura crenulata* with their symbionts were collected from Naples Reef, a subtidal reef lying approximately 20 km west of Santa Barbara. The sea star *Dermasterias imbricata* was collected from under the Signal Oil company pier at Ellwood, California. At Friday Harbor, Washington, the sea star *Petalaster* (=*Luidia*) *foliolata* was dredged from Bellingham Bay and the sea cucumber *Stichopus californicus* was dredged from East Sound, Orcas Island. Additional *Stichopus* and all other organisms employed in experiments at Friday Harbor were collected by diving in the vicinity of San Juan Island.

All experimental organisms were maintained in running sea water aquaria. Test organisms were replaced about every 2–3 weeks and no excessive mortality of either worms or their hosts occurred during this time. No worms were used more than once in a particular experiment; however, several experiments utilized the same worms. Worms were removed from their hosts and placed in individual dishes of filtered sea water for at least one hour prior to testing. The water in the dishes was replaced at least once during this period to remove any traces of host secretions. This was found to be necessary particularly with the worms from the mollusc host, since these worms frequently were covered with host mucous. The worms were then introduced into the Y-maze (with test solutions flowing) and their distribution recorded after a ten minute experimental period. A worm which had not entered either arm of the Y (enter = an arbitrary $\frac{1}{3}$ worm's length in the arm) was scored as a negative.

Providing no bias exists in the choice apparatus and each trial is independent of any other trial, the distribution of the worms in the two arms of the maze will approximate a binomial distribution with $P = q = 0.5$. The probability of getting the observed distributions of the worms in particular experiments has thus been evaluated by a chi-square goodness-of-fit analysis. The χ^2 value is the sum of the χ^2 values for the distribution of worms in each arm of the maze, assuming an expected value of 50% of the total number of worms choosing. The probability associated with this χ^2 value is then found by entering the table of χ^2 at 1 degree of freedom.

RESULTS

Chemotactic responses to test effluents

Host recognition. Host recognition behavior of *Arctonoe* was monitored by analyzing the responses of the experimental populations of this symbiont to test effluent in the choice apparatus. In this series of experiments worms were simultaneously exposed to filtered sea water and to effluent from their respective hosts. These results, tabulated in Table I, clearly indicate that except for the population of *Arctonoe* living with the sea star *Petalaster,* all of the experimental populations of this symbiont exhibited a significant preference for the arm of the Y containing water in which their original host was being maintained. The worms from *Petalaster* not only failed to show any overt recognition of the effluent from their host but also exhibited no response which could be interpreted as recognition when offered tubefeet excised from this star or sponge swabs bathed in host mucous.

Specificity of the host recognition response. Four of the five experimental populations exhibited a statistical preference for test solutions from their respective hosts. The specificity of these responses was investigated in a series of experiments in which symbionts from the various host populations were presented with test effluents from organisms closely related to the symbiont's original host or organisms which have been reported as being alternate hosts for *A. pulchra.* Again, worms were exposed to filtered sea water and the test effluents simultaneously.

The results of the experiments for the determination of the specificity of the responses of populations of *Arctonoe* are presented in Table II. The general pattern of the responses is one of rather pronounced specificity which is evident in the responses of the worms living with *Dermasterias* and the two species of *Stichopus.* These worms exhibited a significant response only towards their original host when assayed under these experimental conditions (Tables I and II). In fact, of the nineteen test situations involving worms from five species of hosts, a significant response to an organism other than a worm's original host occurred in only three tests. The first of these involved worms from the sea star *Petalaster* which although they did not respond to their original host (Table I) did respond to another asteroid, *Solaster stimpsoni* (Table II). This is the only incidence of a response by these worms which could in any way be construed as being indicative of "recognition." Finally, worms from the gastropod *Megathura* exhibited two responses which deviated from the general trend of specificity which seems to characterize the host recognition behavior of *A. pulchra.* In one test these worms responded very significantly to an alternate host, the holothurian *Stichopus parvimensis,* in addition to their original gastropod host (Tables I and II). In another test these same worms responded to the gastropod *Haliotis* (Table II); however, since only a few

TABLE I

The responses of Arctonoe pulchra *to effluents from its respective hosts*

Original host	Distribution			χ^2 Host *vs* Blank	*P*	Total # exps.
	Host	Blank	Negative			
Asteroidea:						
Petalaster foliolata	16	16	13	0.0	1	4
Dermasterias imbricata	63	5	21	49.4	<0.005	7
Holothuroidea:						
Stichopus californicus	26	2	9	20.6	<0.005	4
Stichopus parvimensis	193	29	51	120.6	<0.005	16
Gastropoda:						
Megathura crenulata	91	15	43	54.4	<0.005	10

TABLE II

The response of Arctonoe *to non-host effluents*

Worms from	Test organisms	Distribution			χ^2 Host *vs* Blank	*P*	Total # exps.
		Host	Blank	Negative			
Petalaster foliolata	Holothuroidea						
	Stichopus californicus	14	11	12	0.36	0.5–0.75	3
	Asteroidea						
	Dermasterias imbricata	7	10	21	0.52	0.25–0.5	2
	Pteraster tesselatus	11	10	21	0.05	0.75–0.9	4
	Solaster stimpsoni	20	9	25	4.16	<0.05	4
Dermasterias imbricata	Holothuroidea						
	Stichopus parvimensis	42	30	75	2.0	0.25–0.5	8
	Asteroidea						
	Patiria miniata	1	1	24	0.0	1	2
	Gastropoda						
	Megathura crenulata	3	4	23	0.14	0.5–0.75	4
Stichopus californicus	Holothuroidea						
	Cucumaria miniata	12	15	22	0.33	0.5–0.75	2
	Asteroidea						
	Dermasterias imbricata	3	7	5	1.6	0.1–0.25	2
	Petalaster foliolata	8	8	23	0.0	1	3
	Pteraster tesselatus	13	8	17	1.19	0.25–0.5	4
	Solaster stimpsoni	20	11	34	2.6	0.1–0.25	5
Stichopus parvimensis	Asteroidea						
	Patiria miniata	4	10	16	2.56	0.1–0.25	2
	Dermasterias imbricata	64	60	33	0.13	0.5–0.75	6
	Gastropoda						
	Megathura crenulata	15	32	23	6.8	0.01–0.05	4
Megathura crenulata	Holothuroidea						
	Stichopus parvimensis	50	9	23	28.4	<0.005	6
	Asteroidea						
	Dermasterias imbricata	12	9	31	0.44	0.5	5
	Patiria miniata	8	5	17	0.7	0.25–0.5	2
	Gastropoda						
	Kelletia kelletia	1	5	24	2.66	0.1–0.25	1
	Haliotis rufescens	15	4	33	6.36	<0.025	3

of the worms offered this test organism made a choice, these results may not be indicative of attraction to this species by these worms.

Discrimination between alternate hosts. The host recognition responses of *Arctonoe* were quite specific when worms were offered a host *versus* blank choice situation. As a further test of the specificity of these responses, another series of experiments was performed in which specimens of *Arctonoe* were simultaneously exposed to effluents from their original host and an alternate host. The results of these experiments are presented in Table III.

These results confirm the observation of specificity in the host recognition responses of these symbionts. In all cases except one the worms exhibited a statistically significant preference for the arm of the Y containing effluent from their original host. The presence of an alternate host in the system apparently did not affect the behavior of these worms. Furthermore, the results of the experiments in which *Arctonoe* from *Megathura* was presented effluents from *Megathura* and *Stichopus* provide additional evidence that these worms respond positively to this alternate host species; the worms distributed themselves randomly in the Y-maze. It should be noted that in these experiments control experiments employing worms from the respective hosts insured that the various test effluents were attractive to the test organisms' own symbionts.

Although no quantitative evaluations were made, there were no obvious behavioral variations among any of the worms employed in these experiments which could be directly attributed to differences in age (size) or sex among the worms. Worms as small as 10 mm responded similarly to those as large as 50–60 mm. Likewise, no obvious seasonal variations in these host recognition responses occurred.

Since the protocol utilized in these experiments attempted to exclude any form of information exchange other than by chemical means between the test organisms and *Arctonoe,* the data from these investigations indicate that certain of the hosts for this symbiont release some substance(s) which acts as an attractant for these symbiotic polychaetes. No critical evidence is available to ascertain whether qualitative and/or quantitative differences exist among the attractants. One might reasonably expect qualitative differences to occur among the attractants since the host recognition responses are quite specific. Also, this specificity is evident even when test effluents are "brewed" for much shorter time periods than the 20–24 hour interval used in these experiments, a fact which might indicate that quantitative differences alone do not supply the requisite information to effect the observed behavioral specificity of these symbionts. In addition, the phylogenetic diversity

TABLE III

Specificity of the responses of Arctonoe pulchra *to effluents from alternate hosts*

Worms from:	Distribution when offered host *vs* alternate host		χ^2	*P*	Total # Exps.
Dermasterias imbricata	Dermasterias 57	Megathura 3	46.6	<0.005	5
	Dermasterias 225	Stichopus 25	160	<0.005	19
Stichopus parvimensis	Stichopus 52	Megathura 8	32.2	<0.005	5
	Stichopus 226	Dermasterias 27	190	<0.005	12
Megathura crenulata	Megathura 19	Dermasterias 4	9.7	<0.005	2
	Megathura 22	Stichopus 18	0.4	0.5–0.74	3

of the hosts involved in these associations might result in there being greater molecular diversity among the attractants.

Effects of previous experience

Specificity in the host recognition responses of *Arctonoe pulchra* indicates that this worm is capable of discriminating its respective host from among an array of alternate host or non-host species. This discrimination implies that these worms possess sensory and/or integrative apparatus which permits this discrimination. Regardless of which parameter of the chemical signal from a particular host organism provides the requisite information to effect this discrimination, one wonders whether this behavior is genetically fixed or whether the previous experience of individual worms could modify their responses. Thus, the question of whether some conditioning phenomenon might affect this host recognition behavior was investigated. In the experiments which follow the assay for an effect of various experimental parameters on the specificity of host recognition consisted of exposing worms from a particular host to effluents from two species of host (their original host and one alternate host) simultaneously in the Y-maze. In all cases the appropriate control experiments were conducted to insure that the test effluents were attractive; otherwise, the experimental procedure followed that outlined in Methods.

Effect of long-term physical contact with an alternate host. Field collection data (Dimock, 1970) indicate that associations between *Arctonoe* and its hosts start early in the life of the symbionts. Could such long-term intimacy with a host have any effect on the host recognition behavior exhibited by adult worms?

Previous experience with *Arctonoe pulchra* had clearly indicated to us that the response of this worm to at least two species of hosts was not significantly altered either by maintaining the worms for long periods in the laboratory under ordinary holding conditions, *i.e.,* on their respective hosts in running sea water, or by keeping the worms completely isolated from any physical or chemical contact with any host. In fact, worms which had been isolated from their host continued to respond significantly and specifically to that host after as much as five weeks' isolation. Thus, we performed a series of experiments to determine if intimate physical contact might modify a worm's response to a particular species of host.

A series of reciprocal experiments involving worms from the sea cucumber *Stichopus parvimensis* and the sea star *Dermasterias imbricata* was performed. Worms from the sea cucumber and the sea star were removed from their host, tested in the choice apparatus for their initial host preference and then placed upon the respective alternate host which previously had been freed of all worms. Thus, worms which had previously been on *Stichopus* were placed on *Dermasterias* and vice versa. These worms were kept on the alternate hosts in the laboratory and their host preference behavior was monitored at intervals over a four-week period. The results of these experiments are presented in Table IV.

The data indicate that, indeed, intimate contact between these worms and an alternate host does affect the subsequent behavior of *Arctonoe*. The results of experiments 1 through 3 in Table IV clearly show that by the end of the experiment, either 3 or 4 weeks, the worms no longer exhibited a significant preference for their original host. In all experiments the worms distributed themselves ran-

Table IV

Conditioning worms from one host to an alternate host

Worms initially from:	Exp. #	Time on alternate host	Distribution			χ^2 Host *vs* Host	*P*
			Dermasterias	*Stichopus*	Negative		
Stichopus parvimensis	1	0 weeks	2	29	10	23.4	<0.005
		3 weeks	8	9	1	0.06	0.75–0.9
	2	0 weeks	4	26	0	10.8	<0.005
		2 weeks	6	18	6	6.0	0.01–0.025
		3 weeks	7	19	2	5.54	0.01–0.025
		4 weeks	10	9	7	0.05	0.75–0.9
	3	0 weeks	2	27	3	21.4	<0.005
		2 weeks	5	20	1	8.74	<0.005
		3 weeks	6	13	5	2.56	0.1–0.25
	4	0 weeks	5	28	1	10.9	<0.005
		2 weeks	21	8	3	5.82	0.01–0.025
Dermasterias imbricata	5	0 weeks	29	3	5	21.1	<0.005
		3 weeks	17	9	5	2.46	0.1–0.25
		4 weeks	6	17	5	5.26	0.01–0.025
	6	0 weeks	22	3	5	14.4	<0.005
		2 weeks	7	15	7	2.9	0.05–0.1
		3 weeks	5	18	1	7.32	<0.01
		4 weeks	4	10	1	2.56	0.1–0.25

domly in the maze. It should be noted, however, that control experiments indicated that the test effluent from each host organism was attractive to "naive," *i.e.*, unconditioned, worms from the respective hosts.

The results of the 4th experiment in this series suggested that this conditioning process could have an even more pronounced effect on *Arctonoe* from *Stichopus*. In this experiment (Table IV, Exp. 4) not only did the worms lose their preference for their original host, but they actually switched and exhibited a statistically significant *attraction* towards the alternate host *Dermasterias*. These data clearly indicate that the worms somehow had become "conditioned" to the new host and thus were attracted to it.

The reciprocal experiments which involved placing worms from *Dermasterias* on *Stichopus* yielded similar results (Table IV, Exps. 5 and 6). Once again the effect was dramatic. In both of these experiments the worms underwent a profound change in their host preference; not only did fewer forms choose their original host, but a significant number of the worms chose the "conditioned" host in preference to their original.

Effect of long-term olfactory exposure on host preference. The observations from the foregoing experiments would appear to indicate that intimate contact between *Arctonoe* and a host species influences the subsequent responses of that worm to host effluent. It thus seemed of interest to determine if actual physical

TABLE V

Effect on Arctonoe *from* Stichopus *of long-term olfactory exposure to* Dermasterias

		Choice—1			P Choice	Choice—2			P Choice
		Stichopus	*Dermasterias*	Negative		*Dermasterias*	Blank	Negative	
Experimental worms	Initial	28	1	1	<0.005	14	13	3	0.75–0.9
	2 weeks	26	2	2	<0.005	11	19	0	0.1 –0.25
	3 weeks	20	8	1	<0.05	11	16	2	0.75–0.9
Control worms	Initial	26	2	2	<0.005	12	14	4	0.75–0.9
	2 weeks	24	2	2	<0.005	15	10	3	0.5 –0.75
	3 weeks	22	5	1	<0.005	12	11	5	0.75–0.9

contact between host and symbiont was necessary to effect a change in worm behavior, or whether this change might be brought about simply by exposing the worms to "host odor" for an extended period. In addition to the biological interest of such a question, a quite practical reason for such a determination exists. If *Arctonoe* is influenced by what it smells, might not its responses to later olfactometer tests be affected by exposure during an earlier test to concentrated test effluent?

Worms from *Stichopus parvimensis* were maintained in individual plastic tubes upon a two-layered platform of fiberglass screen suspended in a 15 gallon aquarium of aerated running sea water. Twelve sea stars, *Dermasterias imbricata,* were held in the lower half of the aquarium by the screened barrier. The two layers of screen were separated 1½ inches by a wooden frame. Thus, the worms in the tubes in the upper portion of the aquarium were exposed to rather concentrated *Dermasterias* "odor" but were not permitted physical contact with these stars. At various times during this experiment the sea water stopped running into the aquarium, at these times the worms probably were exposed to an even higher concentration of this alternate host's odor. A control group of *Arctonoe* was held under similar conditions except that no sea stars were placed with them in the aquarium. The experiment was run for three weeks.

The effects of these experimental conditions on the subsequent behavior of *Arctonoe* were monitored in two ways. The first assay was identical to that used in the previous experiments, *i.e.,* worms were exposed to effluents from their original host, *Stichopus,* and the alternate host, *Dermasterias,* simultaneously. The second assay was similar except that effluent from *Dermasterias* was offered to the worms simultaneously with filtered sea water. The work of Thorpe and Jones (1937) suggests that although test organisms might maintain a preference for their original host when offered the original and the "conditioned" host simultaneously, presentation of the "conditioned" host and a "blank" in the olfactometer might detect some effect of the exposure to the alternate host. The results of these analyses are summarized in Table V.

The response of the experimental group of worms to the *Stichopus-Dermasterias* choice situation following three weeks exposure to *Dermasterias* effluent differed significantly from the initial response of this group ($\chi^2 = 4.99$, $P < 0.05$ by a 2×2 contingency table with Yates' correction factor). However, the response of the experimental group at three weeks did not differ significantly from that of the control worms at three weeks ($\chi^2 = 0.002$ by the same test). Both groups of worms exhibited a significant preference for *Stichopus*. In addition, an analysis of the data from the *Dermasterias*-blank choice situation yielded no discernible effect of the continuous olfactory exposure on the behavior of the worms, in spite of the fact that this test is perhaps more sensitive than the two-host choice situation. Thus, the results indicate that the long-term olfactory exposure had no effect on *Arctonoe*. Therefore, it is unlikely that exposure of *Arctonoe* to concentrated effluents in one test affects the subsequent behavior of this worm.

Discussion

Regardless of where in the stimulus-response chain the integration of information necessary to effect the observed behavioral specificity of *A. pulchra* occurs, the data from this study clearly indicate that, indeed, several populations of this symbiont differ in their host recognition behavior from that of conspecifics. Of the five populations of this symbiotic polychaete investigated, the group living with the mud-star *Petalaster foliolata* was singular in its lack of overt host recognition. These observations agree with earlier results obtained by Davenport (1950) for worms from this sea star. However, in this study, unlike Davenport's, only intact, apparently healthy mud-stars were used. Thus, although Davenport's suggestion that injured stars may release some "injury substances" which inhibit the worm's response may be true, such a mechanism cannot be invoked to explain the lack of response of these worms observed in this study. It is possible that associations between this star and *Arctonoe* do not occur as the result of some active host recognition behavioral mechanism. These mud-stars appear to provide the predominant solid substrate in their soft mud habitat, and, if larval or juvenile worms were first attracted to this total environment (Laing, 1937), associations with this host star might come about as a result of random encounters between the worms and this "substrate."

The other experimental populations of *Arctonoe* exhibited statistical preferences in the Y-maze which clearly indicated host recognition. These host recognition responses furthermore seem to be quite specific. Worms from the sea star *Dermasterias* and the two species of *Stichopus* failed to respond to test organisms closely related to their original hosts or to species which function as alternate hosts for this symbiont, hosts which in turn are attractive to their respective symbiotic partners. The observed specificity of this host recognition was maintained even when symbionts were offered a choice between alternate hosts simultaneously (Table III).

There were, however, several exceptions to the observed specificities in the responses of these symbionts. One notable exception was the response of worms from the mud-star *Petalaster* towards the asteroid *Solaster stimpsoni* (Table II). As previously indicated this asteroid has been reported as being a host for *A. pulchra*, but the significance of the observed response of these worms is at present not clear.

The other exceptions to this overall trend of pronounced specificity in these host recognition responses occurred within populations of *Arctonoe* living with the gastropod *Megathura*. The responses of these worms to the gastropod *Haliotis rufescens* (Table II) might suggest that these worms simply were responding to some generalized molluscan attractant. However, the high proportion of worms not making any choice when exposed to this test organism and the lack of response by these worms to the gastropod *Kelletia,* indicate that this is not the case. Further investigations must be undertaken to determine if in fact this observed response is indicative of an attraction to this mollusc.

The responses of *Megathura* worms to effluents from the alternate host *Stichopus parvimensis* provide the most puzzling results of the experiments utilizing these symbionts. This alternate host species, among all of the test organisms, was the only one which evoked a significant response from a population of *A. pulchra* that also responded to its original host. The response to *Stichopus* was further verified by the behavior of these worms when exposed simultaneously to their original host, *Megathura,* and *Stichopus* (Table III). Under these conditions the worms responded equally to the test organisms, *i.e.,* they distributed themselves randomly in the arms of the Y-maze. The significance of such behavior is at present unknown.

Experiments described above clearly indicate that a population of worms may be conditioned to an alternate host. Inherent in most learning theory is the concept of reinforcement. However, Thorpe and Jones (1937), in a study of host selection in parasitic insects, concluded that simple exposure to a certain chemical environment at some time during an organism's life cycle tended to increase responses to those chemical stimuli in subsequent laboratory tests. The nature and function of this olfactory conditioning in insects were explored in a subsequent series of investigations (Thorpe, 1938, 1939, 1963).

Olfactory conditioning has not previously been reported for annelids. Indeed, among the polychaetes no form of learning other than habituation has been conclusively demonstrated except perhaps maze learning (Evans, 1965, 1966*a*, 1966*b*; Wells, 1968). Therefore, the results of the present study are of interest since they show an additional capability for plasticity in the behavioral repertoire of these annelids.

Extensive experimental analyses must be performed before much can be said about the mechanisms involved in effecting this conditioning. The data from the present study suggest that close physical contact may be required to effect this change in olfactory response. Continuous exposure to the effluent from *Dermasterias* had no discernible effect on the behavior of *Arctonoe* (Table V). Wood (1968) found the same thing to be true regarding conditioning of the oyster drill *Urosalpinx*. The lack of effect of this exposure may, however, simply be a function of the time course employed in these experiments. Physical contact with the host may be a more effective means of bringing about conditioning. Monteith (1955) found physical contact more effective for conditioning an insect than olfactory exposure.

Regardless of the mechanisms involved in effecting conditioning this phenomenon is perhaps of fundamental significance to these symbionts. Once an intimate association between a worm and a host is effected, the worm could become conditioned to some chemical signal from the host, a signal which might serve to bind the partners together. If for instance young worms were initially attracted nonspecifically to all of the hosts in a particular habitat, they might then develop specificity in their host recognition behavior as a result of this conditioning phenomenon. At present one may only theorize about the adaptive significance of this capability for modification of the host recognition behavior of these symbionts. Further analysis of the role of conditioning in the life cycle of these organisms will await rearing of the worms and a determination of what parameters effect the onset of host recognition behavior and the specificity in these responses exhibited by this polychaete.

Recent investigations in our laboratory (Dimock, 1970) have indicated a possible role for the host recognition responses of adult *A. pulchra*. The distribution of large worms (>20 mm) on two of the hosts of this symbiont, *Stichopus parvimensis* and *Megathura crenulata,* is very regular at one large worm per host. Furthermore, experimental evidence clearly indicates that these large worms can regulate this density, apparently through intraspecific aggression. Thus, since small worms are present at much higher densities on these hosts than are large, some worms may at some point in time be forced to leave a host organism and colonize another. Certainly, an obligate symbiont must find another host or die if it for any reason becomes separated from its host. Therefore, the possession of some mechanism for effecting host recognition and relocation may be of selective advantage to this symbiont.

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SUMMARY

1. Several populations of the symbiotic polychaete *Arctonoe pulchra* were examined with regard to the ability of these worms to detect chemical signals emanating from their hosts. Four of the five experimental populations exhibited chemotactic responses which can be interpreted as host recognition.

2. The host recognition responses were for the most part very specific in that worms consistently chose their original host when presented effluents from a variety of organisms in a Y-maze choice apparatus.

3. The specificity of these chemotactic responses could be affected by the previous experience of individual worms. That is, worms could be conditioned to respond to an organism which previously was unattractive to them.

4. The mechanisms involved in effecting this modification of behavior are not clear. However, the results of this study indicate a heretofore unknown behavioral capability among these polychaetes.

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PETROLISTHES TRIDENTATUS: THE DEVELOPMENT OF LARVAE FROM A PACIFIC SPECIMEN IN LABORATORY CULTURE WITH A DISCUSSION OF LARVAL CHARACTERS IN THE GENUS (CRUSTACEA: DECAPODA; PORCELLANIDAE)[1]

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Petrolisthes tridentatus Stimpson, 1858, is a diminutive porcellanid species which is apparently confined to the littoral zone on both sides of the Panamanian isthmus. In the tropical Atlantic it is found throughout the Caribbean region from the Bahama Islands to Trinidad. In the Pacific it has been collected sporadically from San Juan del Sur, Nicaragua to Isla Puna, Ecuador. Other than collection data nothing is known of the biology of the species and the larval development is completely undescribed.

The purpose of this paper is to describe the complete larval development, from hatching to megalopal stage of larvae from a Pacific specimen of *P. tridentatus*. In some other amphi-Panamanian species, the larvae obtained from adults inhabiting one side of the isthmus have differed considerably in morphological features from larvae obtained from adults on the opposite side (Gore, 1971; 1972a in press). Such may prove to be the case in *P. tridentatus* when larvae described herein are compared with those reared from an Atlantic specimen.

Materials and Methods

An ovigerous female collected from Punta Paitilla, Panama on 31 December 1968 was shipped by air to the Rosenstiel School of Marine and Atmospheric Science (RSMAS) where it was isolated in a 19 cm diameter glass bowl filled with nonflowing seawater. Hatching occurred on 8 January 1969.

A series of 120 larvae were cultured in 24-compartmented plastic trays using methods previously described for other larval cultures (Gore, 1968, 1970, 1971). Individual zoeae were placed in each compartment. Each compartment was filled with about 80 ml of filtered Biscayne Bay seawater. Salinity varied from 32–34.7‰ throughout the rearing experiment. Water was changed in the trays every day at temperatures of 20° C and higher; every other day at lower temperatures. Larvae were fed *Artemia salina* nauplii in amounts sufficient to ensure that excess nauplii remained in each compartment as noted at times of water change. The series was cultured at average temperatures of 10, 13.5 (±0.5°), 20, 24.8 (range 24–25.5° C) and 29.6° (range 28–32°) C in controlled temperature units (CTU). The temperature fell for one day in the 29.6° C CTU to 26° C; this value is included in the average value computed for that temperature. These values are expressed as 5° increments in Table I. All measurements were made with a Lafayette slide micrometer. In the zoeae, carapace lengths were measured from the anterior margin of the eyes, to the points of insertion of the posterior carapace spines. In the megalopae, carapace lengths were measured from the frontal regions to the posterior edges of the carapaces; carapace widths were measured across the widest part of the carapaces. The sizes given are the arithmetic average for the number of specimens examined.

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The spent female and a complete larval series are deposited in the museum of RSMAS; UMML 32: 4365, 32: 4366.

Results

Rearing experiment

Petrolisthes tridentatus hatches as a pre-zoea and remains as such for approximately two hours. Two subsequent zoeal stages and a megalopal stage follow. From the data presented in Figure 1 and Table I it is seen that the first zoeal stage lasted from three to six days; the second zoeal stage lasted from five to 11 days, and the megalopal stage lasted from seven to 17 days. Duration of the stages is apparently temperature dependent. *Petrolisthes tridentatus* is able to complete its larval life cycle in the laboratory in as little as two weeks at 29° C and usually less than a month at 20° C. Crab stages were obtained at 20° C and higher. At 29° C the larval duration was shortest but mortality was highest; less than 50% of the larvae survived to attain megalopal stage and only two crab stages were obtained. At 25° C 50% of the surviving megalopae attained crab stage. At 20° C 83% of surviving second zoeae reached megalopal stage but nearly all subsequently died. At 20° C crab stages were obtained from 11 and 17 day old megalopae. It appeared

Table I

Petrolisthes tridentatus: *duration of larval life in days at various temperatures*

		Minimum	Mean	Maximum	Total number molting to next stage
10° C		Did not progress beyond first zoeal stage			
15° C		Did not progress beyond first zoeal stage			
20° C	Zoea I	5*	5	6	23
	Zoea II	9	10*	11	21
	Megalopa	11	14	17	2
25° C	Zoea I	4*	4	4	20
	Zoea II	5	6*	6	20
	Megalopa	8	10*	13	12
30° C	Zoea I	3*	3	4	13
	Zoea II	5*	5	6	10
	Megalopa	7	No Value	8	2

* = Most frequent value.
No value = No mean due to mortality of larvae.

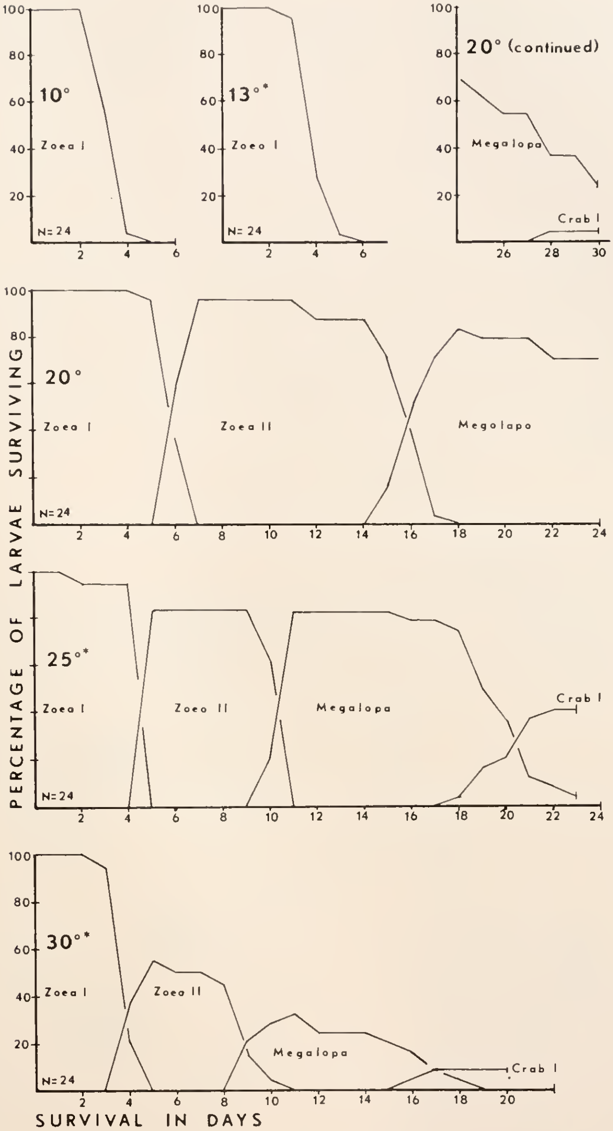

FIGURE 1. Percentage and duration of survival of larvae of *Petrolisthes tridentatus* Stimpson, reared under laboratory conditions. N is the number of larvae reared at each temperature (C°) in each series. The asterisk indicates the temperature rounded off to the closest whole number (see text for explanation).

that 25° C was the most favorable temperature in this series at which to culture larvae of this species.

Molting from stage to stage throughout the series was regular and occurred in a one to two day period, except at 20° C where the megalopal stage was reached by zoeae in the series over a four day period. Contrary to results noted in previous studies (Gore, 1968; 1970, 1971) the molt to megalopa did not appear to be a critical period and all surviving zoeae in the series passed into this stage with no apparent difficulty.

The drop in temperature from 30 to 26° C occurred the day prior to the molt to megalopal stage. Of 11 surviving second zoeae, eight successfully completed the megalopa molt; of these, two progressed to crab stage I. All megalopae and exuviae from this temperature were examined but no noticeable variation either in morphology or number and position of major setae was noted. What effect the temperature drop had on the duration of the megalopal stage is unknown. However, two crab stages were obtained after remaining as megalopae seven and eight days, respectively. Two other megalopae died in molt to the crab stage, one after eight days and the other after nine days as a megalopa. This suggests that the ability to molt was little affected since megalopae reared at other temperatures which remained more or less constant also molted over a two to four day period.

DESCRIPTION OF THE LARVAE

Zoea I

Carapace length: 1.35 mm.
Number of specimens examined: 8.

Carapace: (Fig. 2, A). Typically porcellanid; rostral spine straight or with noticeable upsweep, about 1.8 times carapace length, armed ventrally and laterally

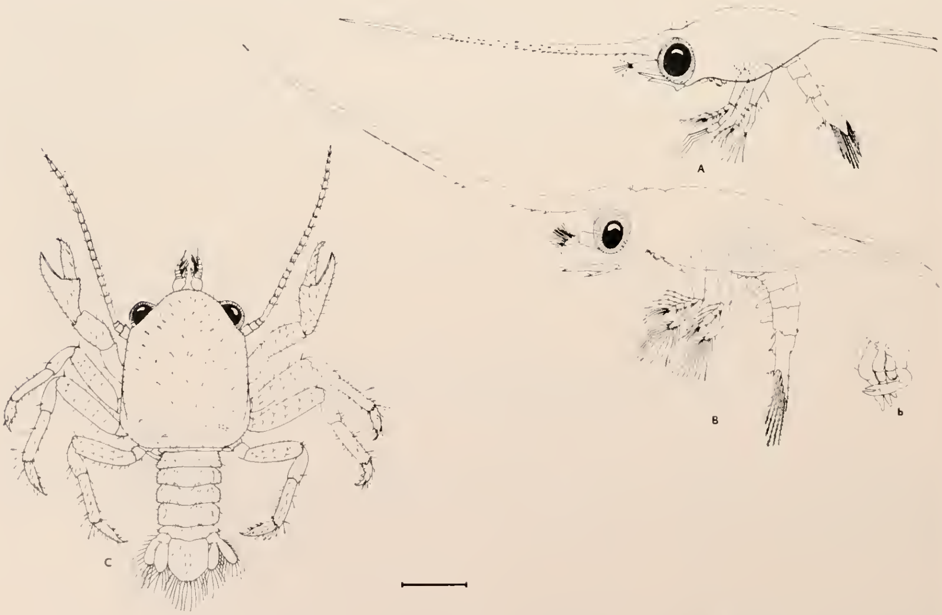

FIGURE 2. The zoeal and megalopal stages of *Petrolisthes tridentatus* Stimpson; A, First zoea; B, Second zoea; b, Detail of early stage pereiopods; C, Megalopa. Scale line equals 0.5 mm.

with scattered spinules, as illustrated; tip naked. Posterior carapace spines about equal to carapace length, each with three to five small nubs ventrally. Lower margin of carapace appears distinctly crenulate under high power (400 ×). Two pairs of setae dorsally above eyes; latter sessile.

Antennule: (Fig. 3, A). Simple rod; three aesthetascs (one subterminal) and three setae, as illustrated.

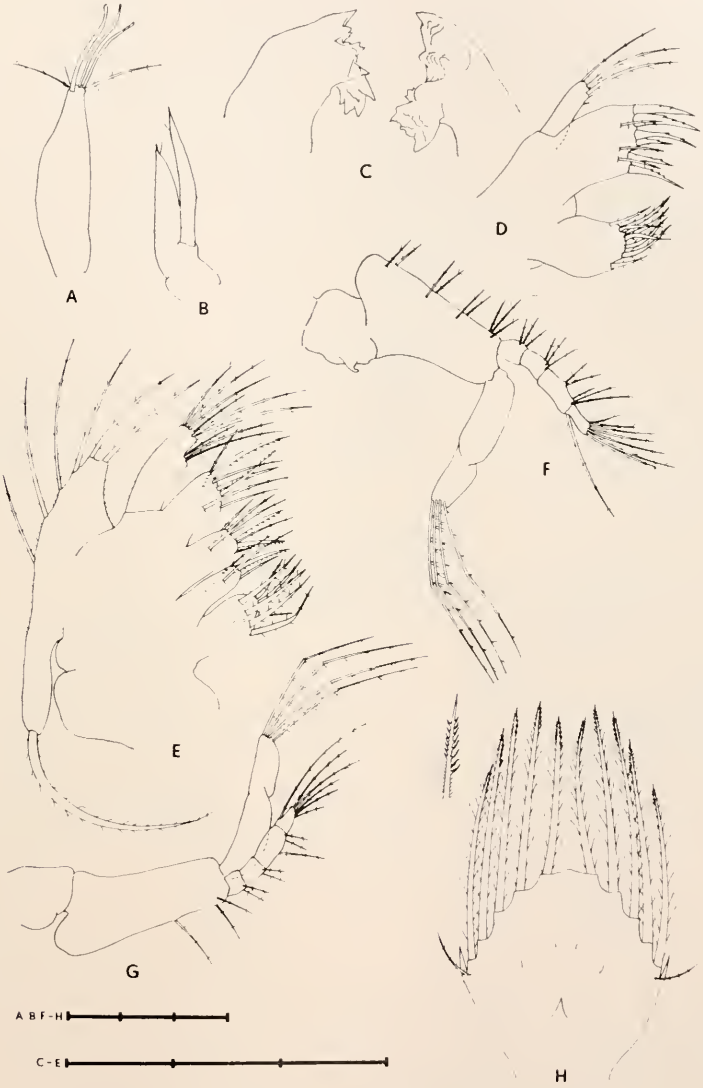

FIGURE 3. First zoeal appendages of *Petrolisthes tridentatus* Stimpson; A, Antennule; B, Antenna; C, Mandibles; D, Maxillule; E, Maxilla; F, Maxilliped 1; G, Maxilliped 2; H, Telson. Scale lines equal 0.3 mm.

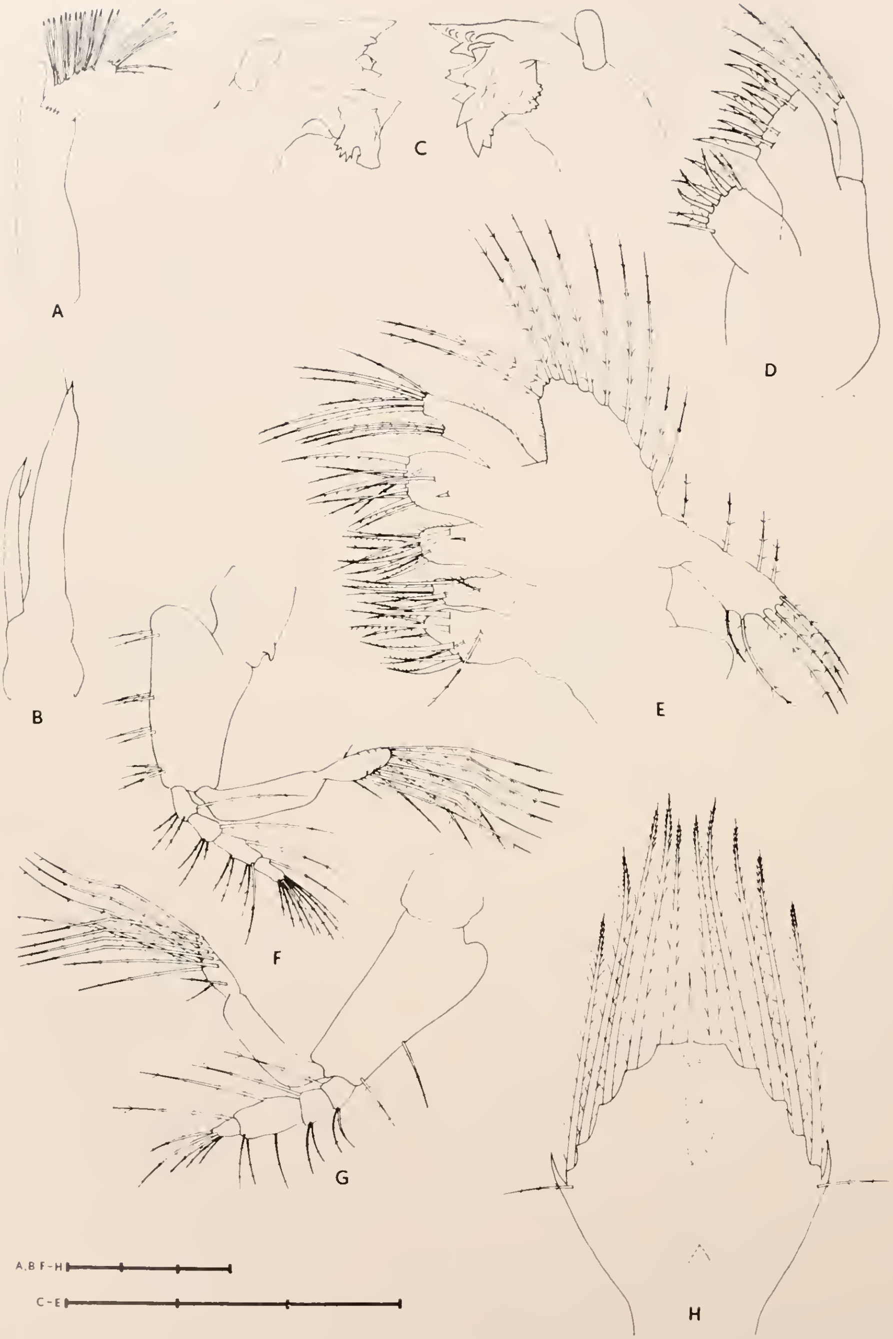
A
B
C
D
E
F
G
H
A,B F–H
C–E

FIGURE 4.

Antenna: (Fig. 3, B). Exopodite about ¼ longer than endopodite, unarmed except for one subterminal seta; endopodite somewhat swollen, drawn into distinct spine, with one subterminal seta.

Mandibles: (Fig. 3, C). Asymmetrical processes, distinctly dentate or with prominent molar process, as shown.

Maxillule: (Fig. 3, D). Endopodite unsegmented, with three setae plus a smaller spinule subterminally. Basal endite with six spines and three setae; coxal endite with six spines and two strong setae, as illustrated.

Maxilla: (Fig. 3, E). Endopodite setae: three terminally, three subterminally, three laterally. Basal endite proximal and distal lobes each with two spines, five setae. Coxal endite proximal and distal lobes with setae as follows: four spines, three setae, and one spine, three setae. Scaphognathite with five to six setae around margin plus one long apical seta, as illustrated.

Maxilliped 1: (Fig. 3, F). Coxopodite naked but with distinct hook-like projection anteriorly, as shown. Basipodite setae 2, 2, 2, 3 progressing distally. Endopodite four-segmented, setae as follows: 3, 3, 2 + 3, 7 or rarely 8 terminally, plus one dorsal seta as shown. Exopodite two segmented, four natatory setae.

Maxilliped 2: (Fig. 3, G). Coxopodite naked. Basipodite setae 1, 2. Endopodite four-segmented, setae as follows: 2, 2, 1 + 2, 5 plus one dorsal seta. Exopodite two segmented; four natatory setae.

Maxilliped 3: Small, undistinguished buds which enlarge slightly as stage progresses. Naked.

Pereiopods: (Fig. 2, A). Amorphous buds at beginning of stage, but gradually assuming form as stage progresses. Incipient segmentation may appear.

Abdomen: (Fig. 2, A). Last three somites with lateral spines, becoming longer and stronger nearer telson.

Telson: (Fig. 3, H). Setae formula 7 + 7, last pair of plumose setae on central prominence. Plumose setae each with hook-like spines, facing inward but more developed on numbers three to five, as shown in detail. Other setae as shown. Anal spine present.

Color: Zoea transparent. Tip and distal ⅔ of rostrum diffusely orange. Posterior spines transparent. Eyes silver-blue in reflected light, this color distinct. Chromatophores as follows: red, laterally on interior of foregut; yellow-red above cheliped buds on carapace; red-orange on top ⅓ of basipodite; yellow on interior of intestine. Labrum, paragnath and mandible tips and maxillule ultramarine blue. Abdominal somites may reflect same blue color.

Second zoea

Carapace length: 1.55 mm.
Number of specimens examined: 10.

Carapace: (Fig. 2, B). Larger, more expanded. Rostral spine about 2 × carapace length, armed ventrally with about six irregularly placed small spinules;

FIGURE 4. Second zoeal appendages of *Petrolisthes tridentatus* Stimpson; A, Antennule; B, Antenna; C, Mandibles; D, Maxillule; E, Maxilla; F, Maxilliped 1; G, Maxilliped 2; H, Telson. Scale lines equal 0.3 mm.

remainder naked. Posterior carapace spines slightly less than carapace length; naked. Two pairs of dorsal setae as shown. Ventral margin of carapace completely smooth, without crenulation. Eyes mobile.

Antennule: (Fig. 4, A). Biramous. Exopodite fused to protopodite, rounded, about $\frac{1}{3}$ endopodite length, naked. Junction of endopodite and protopodite with four small setae. Endopodite with aesthetascs progressing distally as follows: 3, 3, 3, 3, 2, 4 terminally plus two or three setae.

Antenna: (Fig. 4, B). Exopodite about $\frac{2}{3}$ length endopodite; with single subterminal seta. Endopodite drawn into spine with one subterminal seta as illustrated.

Mandible: (Fig. 4, C). Similar to stage I, but larger and with more complex dentition. Molar and incisor processes as shown; each with distinct palp.

Maxillule: (Fig. 4, D). Endopodite a single segment, with three setae terminally; basal endite with seven spines, three setae; coxal endite with six spines, three setae.

Maxilla: (Fig. 4, E). Endopodite setae unchanged from stage I. Setae on basal endite as follows: distal lobe, three spines, seven setae, one small spine; proximal lobe, three spines, five setae, one small spine. Setae on coxal endite: distal lobe, three spines, five setae; proximal lobe, five spines, three strong and three thinner setae. Scaphognathite with 21 setae around margin, with two on the apex, well developed.

Maxilliped 1: (Fig. 4, F). Coxopodite naked, hook-like projection retained; basipodite setae 2, 2, 2, 3; endopodite setae now 3, 3, 2 + 3, 7 – 8, plus dorsal setae as illustrated. Exopodite two-segmented with a total of 12 setae.

Maxilliped 2: (Fig. 4, G). Coxopodite naked; basipodite setae 1, 2; endopodite setae 2, 2, 1 + 2, 5, plus dorsal setae on each segment; as illustrated. Exopodite two-segmented; 12 setae as illustrated.

Maxilliped 3: More developed than previous stage, with endopodite and exopodite more elongate.

Pereiopods: (Fig. 2, b). As illustrated, well developed appendages; segmentation nearly complete. Fifth pereiopod tucked between cheliped and walking leg 1, as illustrated.

Abdomen: (Fig. 2, B). Little changed from first stage except larger; lateral spines remain. Pleopods on segments 2, 3, 4, 5.

Telson: (Fig. 4, H). Fifth pair of plumose setae remain on telson prominence but median spine added. Other setae and armature on long plumose setae as in stage I.

Color: Similar to stage I. Chromatophores as in stage I. Blue color on mouthparts and abdomen still quite intense.

Megalopa

Number of specimens examined: 10.
Carapace length × width: 2.25 × 1.25 mm.

Carapace: (Fig. 2, C). Truncately oval, moderately inflated, smooth to very lightly punctate, sparsely covered with hairs. Frontal region strongly deflexed, not trilobate or tridentate, rounded anteriorly, sparsely covered with hairs. Posterior orbital angle without teeth or spines, rounded. This stage bears little resemblance to an adult. However, some first crab stages exhibit distinctly trilobate frontal region as seen in adult crabs.

Antennule: (Fig. 5, A). Biramous; peduncle three-segmented, basal segment enlarged and inflated with one or two small teeth on outer anterior margin and setae as shown; third segment much inflated with one or two setae. Lower ramus indistinctly seven-segmented, six distinctly so, proximal segment incompletely; aesthetascs on segments two through five in the following sequence of rows and numbers: one row (6), two rows (6, 3 – 4 + 2 setae), two rows (3, 2 + 1 seta), one row (3). Other setae on tip as illustrated. Upper ramus of three distinct segments, but last incompletely divided as to suggest four segments; setae appear as illustrated.

Antenna: (Fig. 5, B). Peduncle three-segmented; flagellum with about three fused segments plus 18 – 22 shorter segments each with about six setae around distal articulation; terminal segment with five long setae as illustrated.

Mandible: (Fig. 5, C). Scoop-shaped processes, appearing heavily chitinized on upper surface of blade. Anterior edge of each appears as illustrated. Each has three-segmented palp: first segment with two spines on outer edge, last with about nine short stout spines.

Maxillule: (Fig. 5, D). Endopodite unsegmented, swollen at base; a short spine and a single seta appears. Coxal endite, lower portion, extended into rounded lobe fringed with fine hairs; a single long seta near its base. Basal endite with shorter seta about midway down its length. Coxal lobe with 11–12 spines, 7 setae. Basal endite with 12–13 short spines, 10 setae.

Maxilla: (Fig. 5, E). Endopodite unsegmented; 2 long setae near tip, one short seta terminally. Coxal and basal lobes heavily spinose and setose, processes difficult to count. Coxal endite with processes on proximal and distal lobes as follows: at least 10 terminally + about 16 encircling lobe; 5 terminally, 7 progressing down the side as illustrated. Basal endite proximal and distal lobes with processes as follows: 12 terminally, 3 to 4 beneath; about 25–28 processes; short stubby spines on lateral surface of each as illustrated. Scaphognathite with about 44 setae around margin plus smaller hairs on lateral surface as illustrated.

Maxilliped 1: (Fig. 5, F). Endopodite and exopodite appear almost unchitinized; 4 short terminal spines plus setae as illustrated on former, 7–8 terminal and 4 lateral setae on latter. Protopodite with about 24 setae on the basal endite and 8 terminal setae on the coxal endite plus others laterally as illustrated.

Maxilliped 2: (Fig. 5, G). Exopodite two-segmented six to eight terminal setae and three lateral spines as shown. Endopodite four-segmented, last two segments heavily spinose, with at least 12 and 10 spines, respectively, first two segments each with about 5 setae placed as illustrated. Basipodite and coxopodite with setae as illustrated.

Maxilliped 3: (Fig. 5, H). Coxopodite with 2 strong distinct spines plus additional setae. Basipodite with setae as shown. Exopodite with three terminal, one lateral, setae. Endopodite five-segmented, first two with lateral blade-like projections. Ischium with total of 12 setae; merus with 13 long plumose setae; carpus with six strong dagger-like spines, 12 long plumose setae; propodus with 8 strong dagger-like spines, nine long plumose setae; dactyl with four strong dagger-like spines and nine long plumose setae. Other smaller setae appear as illustrated.

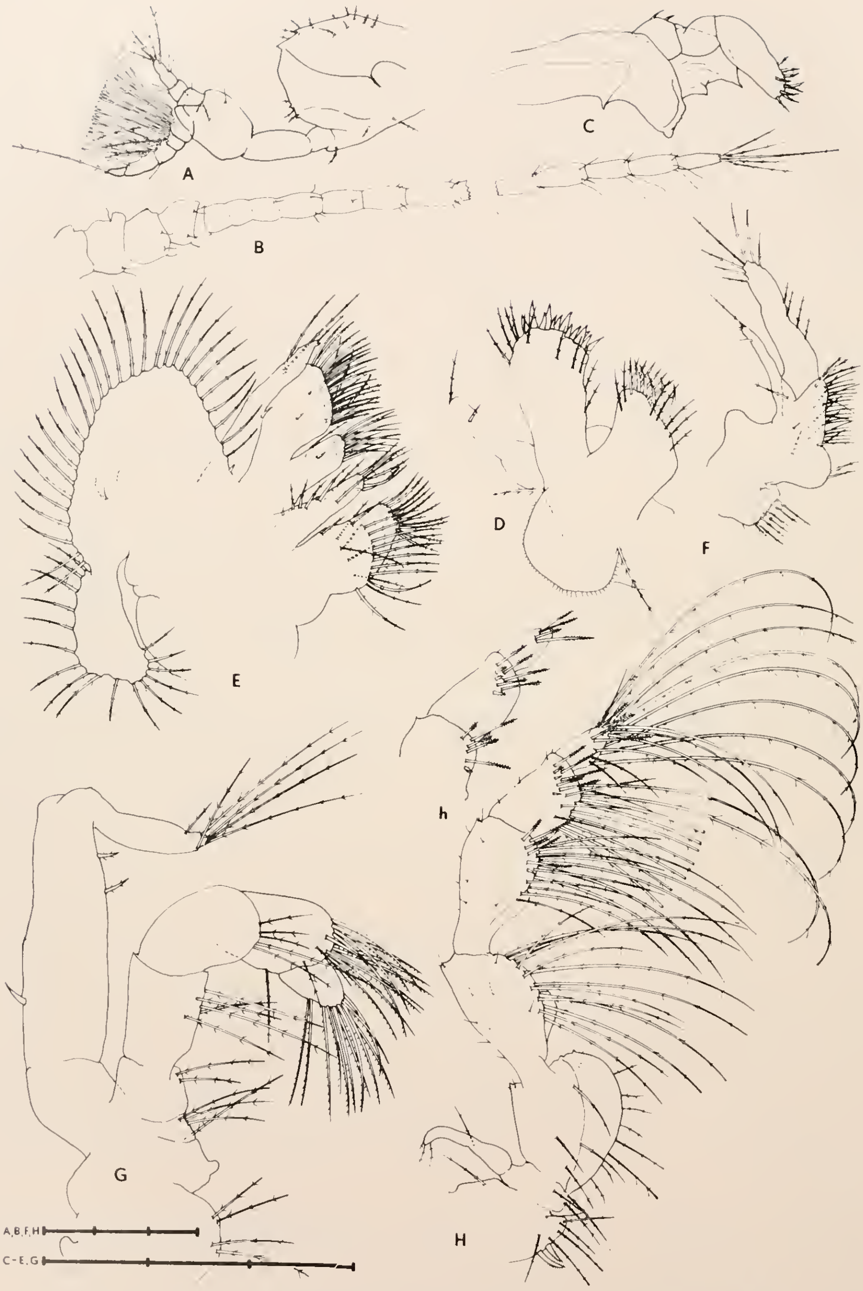

Figure 5. Megalopal sensory and feeding appendages of *Petrolisthes tridentatus* Stimpson; A, Antennule; B, Antenna; C, Mandibles; D, Maxillule; E, Maxilla; F, Maxilliped 1; G, Maxilliped 2; H, Maxilliped 3; h, Spine position on maxilliped 3. Not all setae are completely figured. Scale lines equal 0.3 mm.

Pereiopods: (Figs. 2, C; 6, A, B, E). Chelipeds not overly large, flattened, somewhat subequal, covered with many setae. Moveable finger of each with two distinct spines laterally as illustrated (Figure 6, E). Carpus of chelipeds appearing unarmed but under high magnification about three very small spines appear on interior edge; postero-distal edge with one or two small spinules. Dorsal margin of propodus next to articulation of dactylus may have two hooked spinules. Merus, carpus and propodus of walking legs as illustrated; merus with lateral setae projecting from rugae as illustrated (Figure 6, A), plus about six very small spinules on dorsal margin; one large distinct spine dorso-distally; carpus with setae as shown plus distinct long spine laterally; propodus with two strong spines placed more or less laterally plus five spines ventrally, in addition to long and short setae illustrated; dactylus with setae and spinules as shown. Pereiopod 5 (Figure 6, B) as illustrated, each with five to six long serrate scythe-like setae.

Pleopods: (Fig. 6, C, D). Occur on segments two-five; biramous, becoming

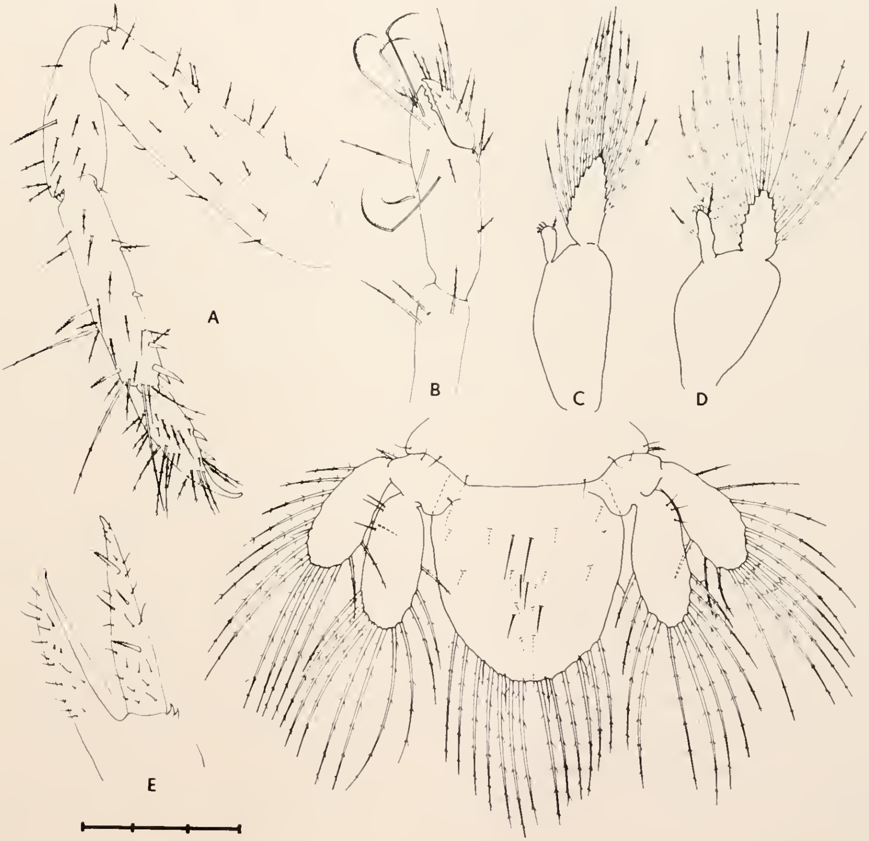

FIGURE 6. Megalopal locomotory appendages and tail fan of *Petrolisthes tridentatus* Stimpson A, Pereiopod 2; B, Pereiopod 5; C, Pleopod 1; D, Pleopod 4; E, Detail of chela; F, Tail fan (ventral view). Scale lines equal 0.3 mm.

wider toward telson. Setae on exopodites most often 13, occasionally 14. Endopodite setae progressing toward telson usually 0, 0, 1, 2, but may vary from 0 to 2 on last two pleopods; all developed with appendix interna.

Abdomen: (Fig. 2, C). Pleura with numerous long setae as shown.

Tail fan: (Fig. 6, F). Six to seven long plumose setae interspersed with two or three shorter setae on each side of telson as shown; numbers not consistent. Uropods biramous, exopodites with about 14 to 16 setae; endopodites with 11 setae; around outer edges. Telson plate with two long distinct setae ventrally plus others as illustrated.

Color: Megalopa transparent. Eyes a distinct sea-foam green. Red chromatophores placed as follows: dorsally next to articulation of moveable finger on propodus of cheliped; dorsally near articulation of carpus with propodus of same; antero-dorsally and ventrally on merus of cheliped. On carapace as follows: frontal region with one just interior to each eye plus one on interior of carapace; lateral margins with about six on each side; hepatic region each with large expanded chromatophore; interiorly in gastric region with very large expanded chromatophore. A small red chromatophore ventrally on second abdominal somite; another on articulation of carpus and propodus of third maxilliped.

Discussion

The zoeae of *Petrolisthes tridentatus* may be recognized in the plankton by the following features: the lower margin of the carapace is distinctly crenulate in the first zoea, the rostral spine in both zoeal stages is relatively short compared to those seen in other porcellanid larvae, and is heavily armed in zoea I and sparsely so in zoea II, there are lateral spines on the last three abdominal somites, and the coxopodite of maxilliped 1 has a noticeable hook-like projection anteriorly. In live specimens the distinct silver-blue color of the eyes and the chromatophore color and position in both stages may aid in identification. The armature on the tips of the plumose setae of the telson is an additional feature which may be useful, but must be used with care since this feature is also seen in other *Petrolisthes* larvae, and in larvae of the genus *Pachycheles*.

The megalopal stage, while definitely porcellanid in character (*e.g.,* reduced fifth legs) does not much resemble the adult. The frontal region is not trilobate in this stage, but usually becomes so upon molt to first crab stage. In addition, there are three small spines anteriorly on the margin of the carpus whereas in the adult the margin is unarmed. Nevertheless, the megalopal stage of *P. tridentatus* has certain features which should enable one to recognize it in plankton collections. The carpus and dactylus of the cheliped, and the merus, carpus and propodus of the walking legs all have at least one strong distinct spine distally; the dactyl of the cheliped has two spines on the lateral surface while the propodus of each walking leg has these spines more or less dorso-laterally. The walking legs also possess several very long setae, as do the dorso-lateral surfaces of each abdominal pleuron. Features on the mouthparts include two distinct spines on the coxopodite of maxilliped 3, the large numbers of setae on the merus and carpus of this appendage, the three lateral spines on the exopodite of maxilliped 2, the single long seta at the base of both the coxal and basal endites of the maxillule, and the spine and seta on the endopodite of the same. Unfortunately, observation of these features requires dissection of the mouthparts, making such features less useful than the overall morphological characters previously described.

In live megalopae the large distinct chromatophore on the gastric region, resembling a splash of red ink, and the smaller chromatophores along the lateral margin should make this species easily identifiable in the plankton.

The zoeal stages of *P. tridentatus* have the fifth pair of telson setae on the central prominence in stage I, and a median spine in this position in stage II. Thus, they conform exactly to Lebour's (1943) classification of such larvae as members of the *Petrolisthes*-group. In addition, the mandibles have a palp in stage II zoeae. This feature appears to be limited to larvae which belong to the *Petrolisthes*-group of larvae (but see below).

The larvae of *P. tridentatus* when compared with larvae of other species of *Petrolisthes* exhibit many features in common. These are summarized as far as available data permit in Table II (see Sankolli, 1967; Shenoy and Sankolli, 1967; Gohar and Al Kholy, 1957; Gore, 1970). Although some descriptions of the larvae which are compared in the table lack much needed detail it is still possible to make several generalizations concerning the larvae of the genus *Petrolisthes* which conform to Lebour's grouping. The larvae of *Petrolisthes platymerus,* and both *P. elongatus* and *P. novaezelandiae* (see Wear, 1964a, 1964b) are excluded since they do not fit into Lebour's category. The first two species have already been compared in a previous study and a third grouping, the *P. platymerus* group, has been tentatively suggested (Gore, 1972b, in press). Larvae in this third grouping, like those in the *Petrolisthes*-group, also possess a mandibular palp in the second zoeal stage. *Petrolisthes lamarckii* and *P. rufescens* (Table II) are species very closely related to each other which occur in the Indo-Pacific region (Haig, 1964). *Petrolisthes armatus,* a species recorded from tropical west Africa and the Americas has also been alleged to occur in the Indo-Pacific but its occurrence there is doubtful. *P. armatus* is related to *P. asiaticus* which is found in the Indo-Pacific and both of these species were synonymized at one time under *P. lamarckii* (see Haig, 1960, page 54), but all are now considered to be distinct species.

Petrolisthes boscii, another Indo-Pacific species, is not as closely related to the three preceding species, nor is *P. tridentatus.*

As indicated in Table II, the larvae of all these species share most of the following features in the first zoeal stage: antennule with three aesthetascs, three setae; antennal exopodite longer ($\frac{1}{4}$ to 2 ×) than endopodite, with one to three thin setae; mandibles without palps; maxillulary endites each with six spines, and basal endite with three, coxal endite with one to three setae, endopodite with three to five setae, often with one small subterminal seta; maxillary endopodite with 3, 2, 3, or 3, 3, 3, setae, next three endites with no more than seven processes each, coxal proximal lobe with four processes, and scaphognathite with five to seven setae.

The thoracic appendages show more variability. Setation of the basipodites differs in each of the species, as does that of the endopodites of the first maxilliped. It is interesting to note, however, that if the dorsal seta (I) on the third segment is moved to the terminal position in *P. boscii* and *P. lamarckii* then a setae formula

TABLE II

Comparison of zoeal characters in five species of Petrolisthes

Zoea I	*P. tridentatus*	*P. armatus*[1]	*P. boscii*[2]	*P. lamarckii*[3]	*P. rufescens*[4]
Antennule	3 aesthetascs 3 setae	3 aesthetascs 3 setae 2–3 setae (P)	3 aesthetascs 3 setae	3 aesthetascs 3 setae	?4 aesthetascs ?3 setae
Antenna					
Exopodite	$\frac{1}{4}$ >endopodite 1 seta	2 × endopodite 2 setae $\frac{1}{3}$ >endopodite (P)	$\frac{1}{4}$ >endopodite 3 setae	2 × endopodite 3 setae	$\frac{2}{5}$ >endopodite "few hairs"
Mandible	No palp	No palp	No palp	No palp	No palp
Maxillule					
Endopodite	3 setae + 1 subterminally	3 setae + 1 subterminally	5 setae + 1 subterminally	4 setae	3 setae
Bsl. endite	6 spines 3 setae	6 spines 3 setae	6 spines 3 setae	5 spines 3 setae	6? spines
Cox. endite	6 spines 2 setae	6 spines (5 P) 1 setae (2 P)	6 spines 1 seta	6 spines 1 seta	6?spines ?3 setae
Maxilla					
Endopodite	3,3,3, setae	3,2,3, setae	3,3,3, setae*	3,3,3, setae	3,2, — setae
Bsl. endite	7,7, processes	7,7, processes	5,6 processes*	7,5 processes*	5,4 processes*
Cox. endite	4,7 processes	4,7, processes	3,4, processes*	4,5, processes*	4,6, processes*
Scaphognath.	5 — 6 setae	5 setae	7 setae	7 setae	4 setae
Maxilliped 1					
Basipodite	2,2,2,3, setae	1,2,2,3, setae	2,1,1,3, setae*	1,1,1,3, setae*	? 2, 1 setae
Endopodite	3,3,2 +3, 7 +I	3,3,2 +4, 9 +I 3,3,2 +3, 7 +I (P)	3,3,2 +4 +I, 7–8*	3,3,2 +4 +I, 7	1,0,3,4, No dorsal seta*
Maxilliped 1					
Expodite	4 natatory	4 natatory	4 natatory*	4 natatory*	4 +2 "short hairs"
Maxilliped 2					
Basipodite	1,2 setae	1,1 setae	1,2? setae	1 seta	? None
Endopodite	2,2,1 +2, 5 +I	2,2,1 +2, 5 +1	2,2,3 +I, 5	1,2,4,5 ?2,2,1 +2, 5 +I†	3,2,1 +3, 4* No dorsal seta*
Exopodite	4 natatory	4 natatory	4 natatory	4 natatory	4 natatory
Maxilliped 3	Small undistinguished buds	Bifid lobe 1–2 setae occasionally (P:no setae)	"biramous buds"	"biramous . . . rudimentary"	"rudiments"
Pereiopods	Amorphous buds	Undifferentiated buds	"Rudiments . . . (as) small buds"	". . . present as small buds"	"rudiments"
Abdomen	Somites 3, 4, 5 with lateral spine	Somites 4, 5 with lateral spine	Somites 4, 5 with lateral spine	Somites 4, 5 with "sharp" spine	Somites 4, 5 with lateral spine
Telson	5th pair setae on prominence; 3–5 with more prominent hooks	5th pair setae on prominence all armed with distinct spinules	5th pair setae on prominence; all armed with "tooth-like spines"	5th pair setae on prominence; all armed with "tooth-like spines"	5th pair setae on prominence?
Zoea II					
Antennule					
Exopodites	3,3,3,3,2,4, +2–3 setae	4,5,3,3,2,3, +2 setae 4,4,3,3,2,3, +2 setae (P)	2,2,2,2,3, + 3 setae	No description available	?10*
Endopodite	4 setae at jct. protopodite	4 setae at jct. protopodite	3-4 setae at jct. protopodite		4 setae at jct. protopodite
Protopodite		1 lateral, 2 basal setae	1 lateral seta		
Antenna					
Exopodite	$\frac{2}{3}$ endopodite 1 seta	$\frac{1}{2}$ endopodite $\frac{2}{3}$ endopodite (P) 1 — 0 seta	$\frac{1}{2}$ endopodite 4 setae*		$\frac{1}{2}$ endopodite No setae
Mandible	Palp present	Palp present	Palp present		Palp present
Maxillule					
Endopodite	3 setae	3 setae	4 setae		3 setae
Bsl. endite	7 spines 3 setae	7 spines 3 setae	?7 spines ?3 setae		?8 processes*
Cox. endite	6 spines 3 setae	6 spines 3 setae	?6 spines ?4 setae		6 spines ?1 setae*
Maxilla					
Endopodite	3,3,3, setae	3,2,3, setae	3,3,3,?2 setae		5,3, setae?
Bsl. endite	10,8 processes	9,8 processes 10,9 processes (P)	9,11 processes?		6,9 processes?
Cox. endite	8,11 processes	6–8, 8 processes 4,8 processes (P)	5,16 processes?		4,9 processes No specific description
Scaphognathite	19 +2 apical setae	16–20 +1 apical seta 14–16 +1 apical seta (P)	24+2 apical setae		14–18 +2 apical setae

Table II—(continued)

Zoea I	*P. tridentatus*	*P. armatus*[1]	*P. boscii*[2]	*P. lamarckii*[3]	*P. rufescens*[4]
Maxilliped 1					
Basipodite	2,2,2,3 setae	1,1,2,3, setae 1,2,2,3 (P)	2,1,1,3, setae		−,1,1,2? setae*
Endopodite	3+1, 3+1, 2+3 +I,7−8+I	3+I,3+I,2+5 +I,11+I 3+I,3+I,2+3 +I,9+I (P)	3+1,0+I,3+I 3+1,4+I†		1,1,1+1,3+I,10†
Maxilliped 2					
Basipodite	1,2 setae	1,1 setae	1,2 setae		0? setae
Endopodite	2+I,2+1,1+2 +I,5+I	2+I,2+1,1+2 +I,5+I	2+1,1+I,1+I +1,4+I†		−,1,1+1,4†
Exopodite	12 setae	12–15 setae	10 setae		12 setae
Maxilliped 3	All more or less rudimentary but increase in size				Rudimentary
Pereiopods	All more or less developed with segmentation seen				"Not functional"
Pleopods	Somites 2, 3, 4, 5	Somites 2, 3, 4, 5	Somites 2, 3, 4, 5		Somites 2, 3, 4, 5
Telson	Median spine present on central prominence of telson		Median "plumose seta" (= spine) on prominence		Median spine present on central prominence, +2 setae

Data from [1]Gore, 1970; and 1972a (in press); [2] Shenoy and Sankolli, 1967; [3] Sankolli, 1967; [4] Gohar and Al Kholy, 1957.

(P) = data of larvae from Pacific specimens.

* = No specific description given, data derived from illustrations.

† = Illustration unclear, most probable situation indicated.

of 3, 3, 2 + 3 − 4, 7 − 9 + I, is seen. Similarly, in the second maxilliped a formula of 2, 2, 1 + 2, 5 + I, is seen. This would then conform to the formula seen in *P. armatus* and *P. tridentatus*, and might be the more probable situation.

Recurring features in the second zoeal stage of the genus *Petrolisthes* are less clear (because of the limitation in data) but the following appear to be more or less consistent; antennule with five rows of aesthetascs plus three or four terminally, four small setae at the junction of the protopodite with the exopodite, usually one long seta just below the endopodite and two short setae on the medial projection of the protopodite; antennal exopodites are now shorter $(\frac{1}{2}-\frac{2}{3})$ endopodites; mandibles have a palp; maxillulary endites add one to three processes on each; processes on maxillary endites increase by one to four; maxillipedal endopodites add one dorsal seta to each segment plus one or more setae terminally; pleopods appear on somites 2, 3, 4, 5; a median spine occurs on the central prominence of the telson.

Many of these same features occur in larvae of *Pachycheles* and *Megalobrachium,* both members of the *Petrolisthes*-group. *Pachycheles* zoeae can be distinguished from known *Petrolisthes* and *Megalobrachium* zoeae by the antennal exopodite which, in the first stage, is armed laterally down its length with three to four small spines in a row (see Knight, 1966; Boschi, Scelzo and Goldstein, 1967; Sankolli, 1967; Gore, 1971). In *Petrolisthes* and *Megalobrachium* only fine hairs occur here. Both *Petrolisthes* and *Pachycheles* possess hook-like spinules on the tips of the elongate telson setae in both zoeal stages while *Megalobrachium* does not. These spinules occur on all five pairs of setae to a greater or lesser degree in known *Petrolisthes* larvae and in the first zoeal stage of *Pachycheles natalensis* (Sankolli, 1967), but only on the first two pairs of setae in other *Pachycheles* larvae. Since *P. natalensis* occurs in the Indo-Pacific it cannot be confused with *Megalobrachium,* a genus endemic to the New World; and it is separated from known *Petrolisthes* larvae by the telson setae features used in conjunction with antennal exopodite characteristics.

SUMMARY

Petrolisthes tridentatus is a shallow water amphi-Panamanian porcellanid crab. The complete development from hatching through meglopal stage for larvae obtained from a Pacific specimen is described and illustrated. The larval development under laboratory conditions consists of a pre-zoeal stage lasting about two hours, followed by two zoeal stages lasting from three to six and five to 11 days, respectively. The megalopal stage lasts from seven to 17 days. Data from the larvae cultured at different temperatures indicate that *P. tridentatus* can complete its life cycle under laboratory conditions in as little as two weeks at 29° C, and usually in less than a month at 20° C.

The zoeal and megalopal stages of *P. tridentatus* exhibit several features, notably on the coxopodite of maxilliped 1, the last three abdominal somites, and the elongate plumose processes on the telson in the zoeal stages, and on the distal segments of the pereiopods in the megalopal stage which may allow these stages to be recognized in the plankton. *P. tridentatus* zoeae also exhibit telsonic features which clearly place the larvae in the *Petrolisthes*-group of larvae established by Lebour.

The zoeae of *P. tridentatus* have several features in common with other known *Petrolisthes* spp. larvae. These features are discussed and compared in an attempt to provisionally delineate larval characters at the generic level. These characters are differentiated from those known to occur in larvae of *Pachycheles* and *Megalobrachium,* the other members presently belonging to the *Petrolisthes*-group of larvae.

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ACTIVITY PATTERNS IN THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE BARNACLE AND THEIR RELATION TO BEHAVIOR[1]

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In the course of working with the central nervous system of barnacles while investigating the mechanism of the shadow reflex (Gwilliam, 1963, 1965), it was noted that most of the nerve trunks displayed persistent rhythmical activity even when the system was completely isolated from any peripheral input. The median photoreceptor could be included in the preparation, but it was not a necessary component of the system that displayed the activity. The fact that barnacle behavior is so markedly periodic (see, *e.g.*, Crisp and Southward, 1961; Southward and Crisp, 1965) leads to the expectation that there may be some demonstrable relationship between the activity of the isolated central nervous system and the behavior of the intact animal.

The idea that centrally determined "programs" serve to direct behavior in the absence of sensory feed-back is now accepted (see Wilson, 1966 for summary). The "command" fibers of the crayfish (Wiersma, 1952; Wiersma and Ikeda, 1964; Kennedy, Selverston, and Remler, 1969 for summary) and certain of the central nervous system cells in the nudibranch *Tritonia* (Willows, 1967; Dorsett, Willows and Hoyle, 1969) are examples of neurons that direct a complex, integrated output event or events. Evidence from the flight system of certain insects (Wilson, 1961) indicates that there is a central oscillator that determines the pattern of motor discharge to the flight muscles, and the rhythmical discharge in the swimmeret motor roots in the crayfish (Ikeda and Wiersma, 1964) is evidence of an oscillator in the crayfish. Indeed, as Bullock (1961, page 51) states: "In principle it should be no surprise to find that a perfectly coordinated sequence of reciprocal activation of antagonistic muscles forming an adaptive action can arise purely centrally."

The work reported here seeks to determine the reality of spontaneous rhythms in the barnacle central nervous system, the patterns of phase relationships in the different nerve trunks, the identity of some of the muscles served by those nerves, and the probable actions of those muscles in terms of the behavior of the intact animal. Another paper will report on the activity of single cells in the central nervous system.

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Materials and Methods

The animals used for most of the work were specimens of *Balanus cariosus* (Pallas) collected from the central Oregon coast. Some observations were made on specimens of *B. nubilus* Darwin collected from the same region.

The central nervous system was exposed as previously described (Gwilliam, 1965) and the simple further step of removing it from the animal was easily accomplished. In most preparations the median photoreceptor was included to serve as a test input device to check continuity in certain nerve pathways. Some preparations consisted of the central nervous system removed from the body of the barnacle, but left attached via the great splanchnic nerves to the adductor scutorum muscle. This involved retaining the terga and scuta with the muscle. Such a preparation permitted recording intracellular junctional potentials from the muscle fibers while monitoring the activity in any of the several nerve trunks. These two kinds of activity could be displayed simultaneously so that the temporal relationships could be observed. Such preparations are referred to as "semi-isolated" in the text.

Recording of nerve trunk activity was accomplished with Pt.-Iridium hook electrodes or suction electrodes, amplified through conventional A. C. pre-amplifiers and displayed on a cathode-ray oscilloscope. Muscle junctional potentials were recorded with 3 M KCl-filled glass micropipettes of 10–30 megohms resistance, and were amplified with a neutralized input capacity amplifier. Permanent records were made by photographing the oscilloscope trace with a Grass kymograph camera. Simultaneous two, three, and four channel recording was used as required, and long term activity was occasionally recorded on an ink-writing oscillograph (Grass Model 7 Polygraph).

Recordings were made in an air-conditioned darkroom where the temperature was maintained at 16–19° C which is warmer than the sea water the barnacles usually experience, but colder than temperatures reached on many sunny days when the animals are exposed.

The medium bathing all preparations was "Instant Ocean" artificial sea water (Aquarium Systems, Inc.). This proved superior to either stored natural sea water or barnacle Ringer's solution (Hoyle and Smyth, 1963) as judged by the longevity of preparations exposed to the various media. Under the above conditions preparations could be made to last for up to 36 hours without elaborate precautions, but were seldom used for more than eight hours.

Results

Figure 1 illustrates a ventral view of the central nervous system of *Balanus cariosus* as it is pinned out for recording. Many of the smaller nerves and branches of major nerves have been omitted, the purpose of the diagram being to locate the nerves recorded from and to show the major features of the system. The nerve trunks are named according to Darwin (1854) except where inappropriate (*e.g.*, "median photoreceptor" instead of "ophthalmic ganglion"). Where a specific nerve used was not named by Darwin we have coined a simple descriptive name. Thus, an unpaired nerve originating from the anterior part of the dorsal surface of the ventral ganglion has been identified as the "mid-dorsal" nerve. A pair of nerves from the anterior ventral surface of the same ganglion have been called

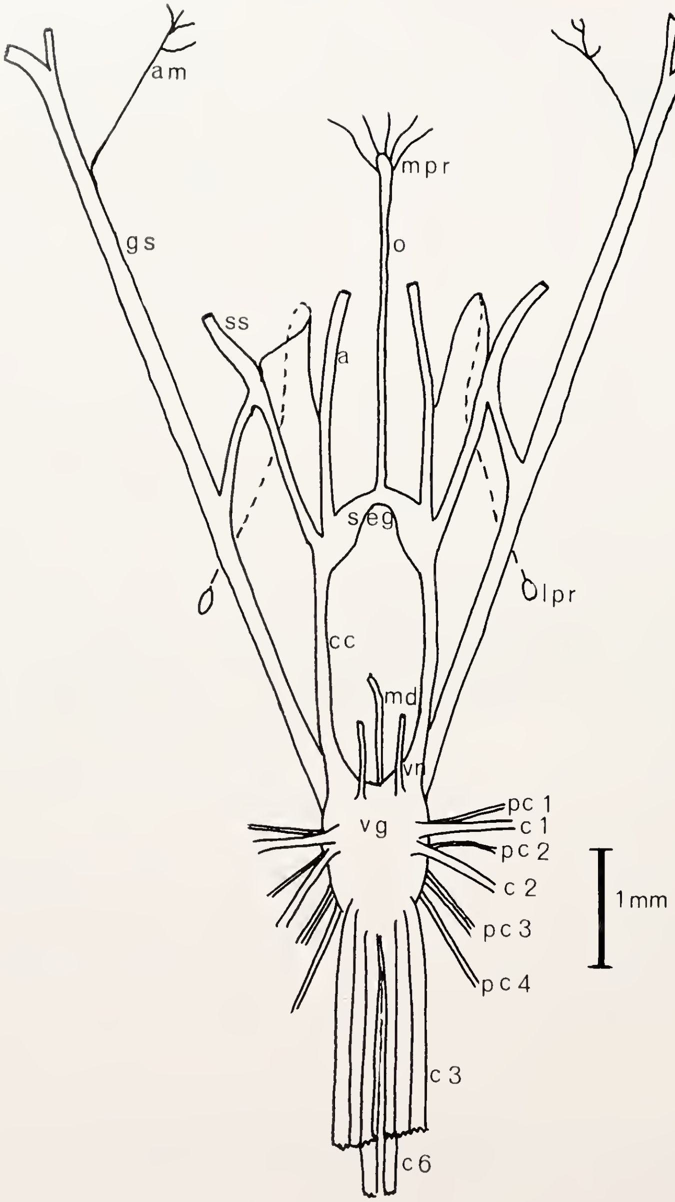

FIGURE 1. Ventral view of the central nervous system of *Balanus cariosus*. The lateral photoreceptors are not included in the preparation used, but are shown for reference. Key to labelling is: am, motor branch to adductor scutorum; a, antennular nerve; cc, circumesophageal connective; c 1–6, cirral nerves; gs, great splanchnic nerve; lpr, lateral photoreceptor; mpr, median photoreceptor; md, mid-dorsal nerve; o, ocellar nerve; pc 1–4, paracirral nerves; seg, supraesophageal ganglion; ss, suprasplanchnic nerve; vg, ventral ganglion; vn, ventral nerves.

simply the "ventral" nerves, and others associated topographically with the main cirral nerves have been called "paracirrals" and numbered in order (Figure 1, pc 1–4). It will be noted that, unlike the situation in those species illustrated by Darwin (1854) and Cornwall (1953), both the first and second cirral nerves are separated from the remainder. Cirrals 3–5 emerge from the ventral ganglion in a group, but the sixth pair are located dorsal to them and are considerably larger, owing in part, no doubt, to the inclusion of the penis nerve in the same sheath.

The connections between the great splanchnic nerve and the suprasplanchnic nerve, and between the suprasplanchnic and the antennular nerve (which, at least proximally, contains the lateral photoreceptor axons) may not always be located exactly as shown, but the general relationships are, in our experience, always as illustrated. Cornwall's interpretation of the innervation of the adductor scutorum muscle as being via his "nerve 4" (the suprasplanchnic of Darwin and Fig. 1) is almost certainly in error. All of the sessile barnacles we have examined have the adductor muscle innervated via the great splanchnic nerve, although the level at which the branch comes off may differ from species to species and indeed, from individual to individual. Further, Cornwall's interpretation of photoreceptor nerves appears to be based in part on Darwin's misconception of the nature of the median photoreceptor (Gwilliam, 1965).

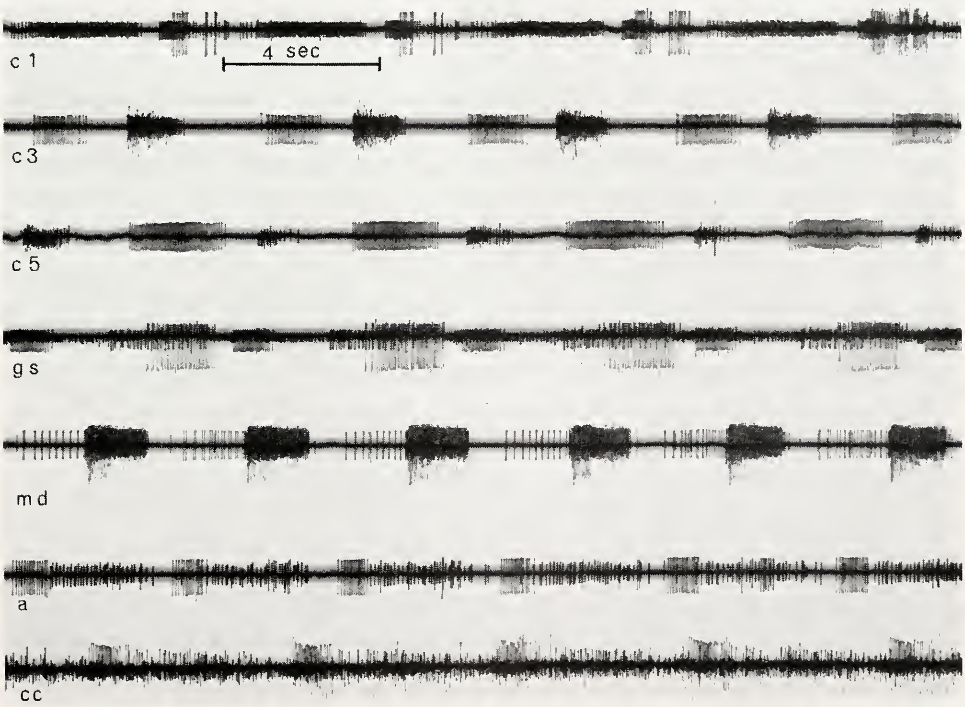

FIGURE 2. Examples of spontaneous rhythmical activity in some nerve trunks of *B. cariosus*. The label at the lower left of each line of recorded activity indicates the nerve trunk from which it was taken. See Figure 1 for identification. Time calibration applies to all records in this figure. Records from several different preparations.

Figure 2 presents segments of filmed records of external recordings from some of these nerves showing spontaneous rhythmical activity in the isolated or semi-isolated central nervous system. Of the major trunks illustrated in Figure 1 only the ocellar nerve and the suprasplanchnic fail to show rhythmical activity. Once the suprasplanchnic is isolated from its connections with the great splanchnic and the antennular, its usual response is complete silence. The ocellar nerve consists principally of sensory axons from the retinular cells but does contain some small efferent fibers (Fahrenbach, 1965) that show no rhythmical activity. All other nerve trunks show rhythms of varying degrees of complexity.

With the possible exception of the first and second (Fig. 2, c 1) all cirral nerves display very similar patterns (Fig. 2, c 3, c 5). The records may appear different from nerve to nerve and preparation to preparation, but almost always consist of a single fiber burst and a multi-fibered burst alternating. The pattern in the first two cirrals is usually more complex and more likely to display random bursts of activity. Sometimes both bursts are prolonged and tend to overlap, but we have never observed them to be completely coincident.

The portion of a record from the great splanchnic nerve (Fig. 2, gs) was taken *en passant* in a semi-isolated preparation, but it is not appreciably different from that seen in an isolated central nervous system. It will be noted that there are at least three different rhythmical fibers, probably a fourth, and a continuous background fiber firing quite regularly. Similarly, the mid-dorsal nerve (Fig. 2, md) has at least three bursting fibers, the patterns of which all overlap.

The antennular nerve (Fig. 2, a) usually displays a rather simple pattern as illustrated but quite often appears to be firing randomly. At other times only the pattern illustrated by the large spikes will be apparent. The circumesophageal connectives (Fig. 2, cc) on the other hand, have quite complex patterns. Usually a major rhythmical burst can be seen, but this is often obscured by much background activity. In this particular illustration the connective was recorded from *en passant.* In cases where the circumesophageal connectives are cut, rhythmical activity is seen only efferent with respect to the ventral ganglion. Isolated supraesophageal ganglia have never, in our experience, shown rhythmical activity of the sort illustrated in Figure 2.

Patterns similar to those seen in isolated and semi-isolated preparations may also be seen in minimally dissected animals. The main difference lies in length of burst and interval between bursts, both of which are usually longer in the more intact animals.

These observations—the consistency and ubiquity of the rhythmical activity in isolated preparations and their presence in minimally dissected animals—suggests to us that the phenomenon is not an artifact of isolation (*cf.* Willows, 1967).

If one accepts the reality of the centrally generated rhythms as a working hypothesis, then one must turn to the normal activities of the intact barnacle to see if there is a correlation between the nervous activity described above and the behavior of the animal. Observations of rhythmical behavior in the intact animal will permit predictions to be made concerning expected patterns in nerves serving particular muscles that can be seen to cause particular movements, and the appropriate recordings made from those nerves.

The most obvious rhythmical behavior a barnacle engages in is "fishing" by extending the body and cirri to form a net and retracting back into the shell. This process must involve not only some muscles acting sequentially, but because extension of the body is accomplished by a hydrostatic mechanism, there must also be sets of muscles acting 180° out of phase with those muscles involved in retraction. In addition to the normal fishing, Crisp and Southward (1961) identify four other kinds of activity. These have been called testing, pumping, fast beat, and extension. Of these, pumping and fast beat are rhythmical. In all of these activities the basic movements are the same, but they vary in extent and frequency. *Balanus cariosus* displays all of these patterns, except that we have not distinguished clearly between a fast and normal beat. Frequency of beat is variable, but the variability shows no discontinuity that would permit other than a very arbitrary division. It is also the case that *B. cariosus* is not as consistently active as many of the smaller species (Southward and Crisp, 1965). In our experience, normal beat occurs only when the animal is exposed to moving water. Testing, pumping, and extension, however, occur in the absence of sufficient moving water to induce normal beat, and these activities start and stop without obvious stimulation.

Given the kind of behavior described above, and if the central nervous activity is a program which directs that behavior, then there should be a variety of "phase" relationships between the various motor fibers serving the muscles involved in extension and retraction. This can be examined by simultaneous recording from various nerve trunks to see if there is a set of events and sequences that lend themselves to this interpretation. A set of positive observations will not, of course, prove the reality of a central spontaneous program, but it is at least consistent with the hypothesis. This has been done for several nerve trunks, and examples of the results are illustrated in Figure 3.

The records shown in Figure 3 reveal both the in-phase and out-of-phase characteristics of the activity seen in the major nerve trunks. In Figure 3, A, the upper trace is from the mid-dorsal nerve, while the lower trace is from paracirral 4. The

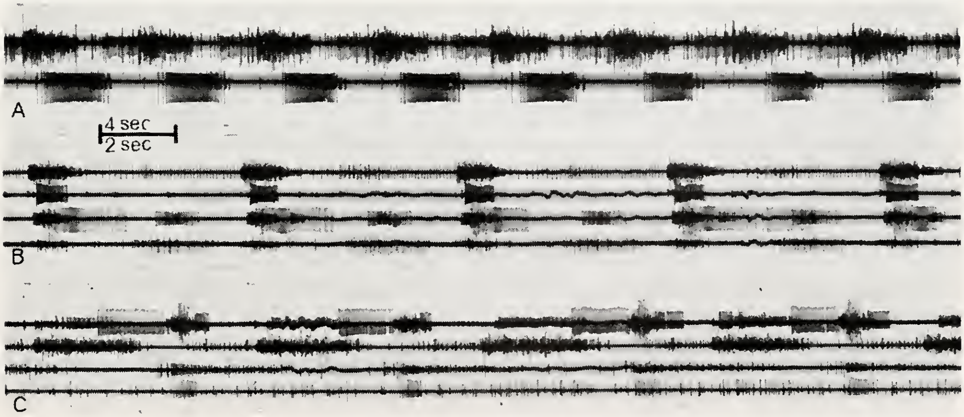

FIGURE 3. Simultaneous recording from various nerves in the isolated central nervous system to show phase relationships; time calibration = four seconds in A, two seconds in B and C; (A.) Top trace, mid-dorsal nerve; bottom trace, paracirral 4; (B.) Top to bottom traces, cirrals 5 and 6; cirral 2; ciral 4; cirral 1; (C.) Top to bottom traces, cirral 4; mid-dorsal; great splanchnic; antennular.

mid-dorsal nerve serves, in part, some of the ventral transverse (dorsal to the nervous system) musculature which exerts pressure on body fluids to bring about an extension of the thorax and cirri. Some fibers from the paracirral nerve serve ventral musculature which is involved in retraction (Gutmann, 1960). In Figure 3, B, all traces are from cirral nerves. It is perhaps significant to note that the principal bursts in two of the nerves serving the three pairs of cirri that make up the actual feeding filter (cirri 4, 5, 6) are just off-set with the posterior cirri leading (lines 3 and 1) as might be expected during a posteriorly originating retraction wave. Cirrals 1 and 2 (lines 4 and 2) have rather different activities in the feeding sequence, and their nerve supply shows somewhat different phase relationships to the others. Care was taken in these recordings to ensure that the timing differences shown could not be due solely to different lengths of conducting pathway based on the assumption that length of nerve from the ganglion was an adequate measure of equal or nearly equal conducting paths. In Figure 3, C, the record from the fourth cirral nerve is from at least four different fibers, and at least three of them are either partially or completely out of phase with the mid-dorsal nerve, while at least one is more or less in phase. A possible explanation of such a record is that the activity coincident and overlapping with activity in the mid-dorsal nerve is from motor fibers activating muscles in the limb base that cause basilar movements during extension. Those out of phase with the mid-dorsal are probably fibers serving the cirral retractor muscles and would be active during withdrawal in the intact animal. The mid-dorsal is out of phase with elements in the great splanchnic and antennular nerves, the latter two showing in-phase bursts. The great splanchnic (Fig. 3, C, line 3) serves (among other things) the adductor muscle and the muscles that depress the mouth cone. These are involved with retraction and closure and would be expected to be out of phase with the mid-dorsal nerves. Similarly, the antennular nerves supply the lateral and rostral scutal depressors. The lateral scutal depressors are concerned, in part, with opening the valves, but the rostrals contract during closure. There is a concerted burst in the antennular in phase with the clearest burst in the great splanchnic, suggesting these are involved in retraction. (The reason for suggesting that the shortest, most clearly defined bursts are associated with the retraction phase is that observations of especially the larger barnacles show quite clearly that extension may be a rather lengthy process, while retraction is usually comparatively rapid.) It is also worth pointing out that muscles involved in increasing hydrostatic pressure and thus causing extension would probably not completely relax. One might therefore expect activity in the nerves serving them to show a regularly varying frequency distribution without ever reaching complete inactivity. Retraction, on the other hand, which is a direct muscular action on the part concerned, might well be discontinuous, or more nearly so. In this connection, discontinuities occur more frequently in the cirral nerves than in the others, and it is the cirri that are served only by retractor muscles (Bullock and Horridge, 1965, page 1181), at least in the distal rami.

Figure 4 presents records that relate some of the activity seen in the isolated nervous system to a behaving motor unit (a single fiber from the adductor scutorum muscle) whose activity could be monitored by observing junctional potentials with intracellular electrodes. The preparation used is that described in Materials and Methods as the semi-isolated preparation. The adductor muscle is very much involved in the rhythmical activity of barnacles in that it is the muscle that closes the valves during the withdrawal-closure reaction.

In Figure 4, A, the upper trace is a recording from the antennular nerve which supplies the rostral scute depressors, while the lower trace is from the adductor muscle. Both these muscles are active during retraction, and the activity in the adductor is virtually coincident with the clearest burst in the antennular. This is what would be predicted if the high frequency burst in the antennular nerve is from the fibers activating the rostral scutal depressor muscles. The secondary burst, of lower frequency, is out of phase with the muscle junctional potentials and may be from those fibers serving the lateral depressors. In Figure 4, B the upper trace is from the great splanchnic nerve, recorded *en passant*. This nerve supplies muscles which are active during extension and retraction. Apart from the adductor muscle and the oral cone depressors, it innervates the large lateral body muscles (numbered 8 by Gutmann, 1960) which are responsible for hauling the body up toward the opercular plates during extension. One would expect, therefore, to find both in-phase and out-of-phase elements in the great splanchnic with reference to adductor contractions, and this is indeed the case.

In the normal behavior sequence the cirri are retracted before the scutes are closed by the adductor muscle. One would expect, therefore, the adductor always to become active toward the end of the cirral burst. Figure 4, C, illustrates the relationship between activity in a cirral nerve (cirral 3) and the adductor scutorum.

Figure 4, D, is from the same sequence as 4, C, but illustrates a neural correlate of a frequently occurring behavioral event observed in intact animals. All barnacle-

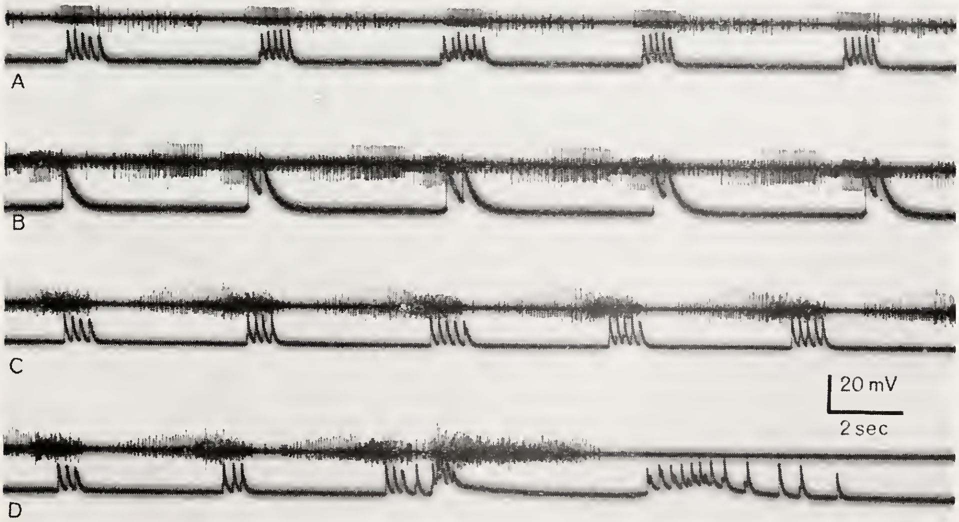

FIGURE 4. Simultaneous recording from various nerves and the adductor muscle (lower trace in all cases). Adductor muscle junctional potentials are D. C. recorded intracellularly. Semi-isolated preparation. Voltage calibration applies to lower trace only; (A.) Antennular nerve, (B.) Great splanchnic nerve (recorded *en passant*), (C.) Cirral 3, (D.) Cirral 3. See text for explanation.

watchers have observed the fact that an undisturbed barnacle will be actively pumping or fishing for a period of time, and then for no obvious reason, undergo withdrawal and closure and remain that way for variable periods. This interruption of activity may itself be rhythmical (Southward and Crisp, 1965) and is commonly observed in most species. For some time we have noted that a particular out-of-rhythm burst of activity, most notable in the cirral and antennular nerves, always preceded a more or less prolonged period of relative inactivity or at least arhythmicity in all of the nerve trunks. Such a burst is seen as the last one in the upper trace in Figure 4, D. It is accompanied by some increased activity in the adductor muscle, but is *followed* by considerable activity and, in this case, an observed massive contraction of the muscle, followed by silence in the cirral nerve. This period of silence and/or arhythmicity lasts for up to five minutes, after which time the characteristic bursting patterns are re-established. Activity very similar to this, but occurring more rapidly, may be induced in such preparations by casting a shadow if the photoreceptor is part of the system.

Observations on many spontaneously active specimens of *B. cariosus* have established that, under laboratory conditions, the beginning of one extension-withdrawal cycle to the beginning of the next one occupies three to seven seconds. Most of the records we have obtained from isolated and semi-isolated preparations of this species show the same periodicity. We have not noted any marked change in periodicity in any of our preparations, but we have not as yet attempted to manipulate environmental factors such as temperature, O_2 concentration, ion balance, or other parameters to see if they have the effect in isolated preparations seen in the intact animals (von Buddenbrock, 1930; Southward and Crisp, 1965).

Discussion

It will be recognized from an examination of the records presented in this paper that the explanations offered for in-phase, out-of-phase relationships and interpretations of sequential events are not rigorously based on a detailed knowledge of the distribution of individual nerve fibers to individual muscles. Recording from multifibered bundles that have wide distributions always introduces considerable uncertainty into interpretation. What is clear, however, is that there are consistent phase and sequential relationships and that the interpretations offered are at least consistent with the known distribution of the nerves. It will probably be possible to record from some final motor branches and demonstrate these relationships more conclusively, but to date the adductor muscle has been the only one to lend itself to this procedure.

This particular preparation, when isolated, does not appear to need triggering in any way to initiate or support the patterned activity. In this it is similar to the crayfish abdominal cord as reported by Ikeda and Wiersma (1964), even though a later paper (Wiersma and Ikeda, 1964) demonstrates that pattern maintenance is also achieved by stimulation of command fibers found in the thoracic-abdominal connectives. It is unlike the locust flight system (Wilson, 1961), which requires continued stimulation, and *Tritonia* (Dorsett, Willows, and Hoyle, 1969), which requires some sensory input in the intact animal or electrical stimulation in the isolated central nervous system to trigger the fixed action pattern. Further indication of a truly autogenic system comes from the fact that the rhythmic activity has been observed to stop and restart some time later completely spontaneously. Cessation of rhythmical activity is preceded by a positive, recognizable event (see Fig. 4) which is itself probably part of the "program." The fact that this aspect of central nervous system behavior corresponds to observed behavior in the intact animal lends credence to the hypothesis that we are indeed observing the neurological basis of behavior in the barnacle.

The preparations described here exclude the possibility that the timing cue for rhythmical activity originates in discharge from peripheral sense organs related to some external event or to the activity of the animal. The possibility that the timing cue is derived from some general level of excitability due to input over sensory fibers cannot be ruled out at this stage, because it is not known what sort of afferent activity is present. The fact that patterned output persists over a period of several hours following isolation, however, argues against such an interpretation.

Behavioral observations on *B. cariosus* indicate that a current of water is needed to initiate fishing. It should be emphasized that the neuromuscular elements and events involved in fishing are the same as those involved in pumping. The difference is in the extent of activity and the frequency with which it occurs. Even in cases where a barnacle is apparently inactive, it is quite conceivable that certain of the same muscles involved in the obvious activities are contracting rhythmically serving a blood circulating function in the absence of a heart (see, *e.g.,* Blatchford, 1970).

Given the present evidence, the best interpretation is that there is a central timing device that must be regarded as "spontaneous" in that it does not depend on externally generated phasic input to determine the periodicity of its output. It is apparent from variations of degree and timing of intact barnacle rhythmical behavior that the output can be altered or modulated. Two possible mechanisms are, a), changes in the immediate environment of the oscillator neurons (*e.g.,* ionic concentration, temperature) as postulated by Mendelson (1971), and b), feed-back from peripheral sense organs, neither of these necessarily operating to the exclusion of the other. There is no direct evidence of the first in the barnacle material, and the second is best demonstrated by the alteration of rhythmical activity seen in the shadow reflex. Work currently going on at University College North Wales, Department of Marine Biology, has established that there are both extension and flexion receptors in barnacle cirri (J. V. Clarke, personal communication), and observations in our laboratory indicate that there is receptor activity fed back to the central nervous system from mechano-receptors associated with the adductor muscle and the opercular muscles. Records from a presumed mechanoreceptor of unknown location have been published (Gwilliam, 1963, Fig. 10). There is as yet, however, no evidence that these receptors modulate on-going rhythmical activity, but it is reasonable to assume they have this effect. It is also apparent that the timer may be turned off, or uncoupled from the rest of the pathway as silent periods in the isolated preparation demonstrate.

It is possible to record from single cells with patterned output in the barnacle ventral ganglionic mass (Gwilliam, 1968) and it is expected that studies of such cells will give more insight into the properties of this particular system.

We wish to acknowledge with thanks a critical reading of the manuscript by Dr. Derek Dorsett which resulted in considerable improvement.

SUMMARY

1. Rhythmical patterns of activity in most nerve trunks of the sessile barnacle, *Balanus cariosus* (Pallas) have been demonstrated to occur in the totally isolated central nervous system at a periodicity consistent with the behavior of the intact animal.

2. When activity in various nerves is compared by simultaneous recording, a pattern of phase relationships is observed that is consistent with the hypothesis that the patterned activity constitutes a program that determines the behavior of the barnacle.

3. The evidence presented suggests that the centrally generated rhythm is autogenic, because in the isolated central nervous system there is no possibility of regular timing cues being made available to central neurons from peripheral sense organs, and no apparent stimulation is required to start and maintain the rhythm.

4. Single muscle fibers in the adductor scutorum muscle attached to the otherwise isolated central nervous system show excitatory junctional potentials with the same temporal rhythm and the expected in-phase, out-of-phase relationships with a variety of nerve trunks including its own supply.

5. It is suggested that observed variation in intact barnacle behavior may be brought about in the system by some direct influence on oscillator neurons and/or sensory feed-back to modulate the extent and timing of rhythmical activity and by uncoupling the timer from the motor output side by turning it off (inhibition) during periods of inactivity. The first of these would also explain frequency variations seen in the isolated preparations in the absence of sensory feed-back.

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POPULATION DYNAMICS AND LIFE HISTORY OF *CREPIDULA CONVEXA* SAY (GASTROPODA: PROSOBRANCHIA) IN DELAWARE BAY[1]

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Crepidula convexa is a relatively mobile calyptraeid limpet with large eggs, direct development, and a protandric sexuality. It is distributed from New England south to the Gulf of Mexico and the West Indies. We report here on a population in Delaware Bay, New Jersey, studied from June 1966 through September 1968. These findings complement previous observations by W. R. Coe (1936, 1938, 1942a, 1942b, 1949) on the biology of this and other species of *Crepidula*.

Coe (1936, 1942a, 1942b) investigated growth rates, sexual transformation, and development of *Crepidula* species on both coasts of the United States. His observations of *C. convexa* were apparently based primarily on Massachusetts animals (Woods Hole) attached on the shells of *Littorina littorea* or on hermit crab-inhabited gastropod shells. He found that *C. convexa* entered the male sexual phase at 3 weeks of age, when 3–6 mm long. In the next or transitional phase, oogenesis was found to occur before spermatogenesis has been completed but the hermaphroditic condition was too transient to permit self fertilization. He found that mated males entered the transitional phase at 6–8 mm when several months old and that, at least in aquaria, males ordinarily move from female to female. In males isolated from females, however, the male phase ended after only a month, but a longer transitional period ensued. The female phase is attained at about 6 months at lengths ranging from about 6 mm to a maximum of 13 mm.

This report is an extension of Coe's work. It defines the relation between protandric sexuality and the sexual composition of the population, emphasizes the importance of male mobility for phoretic dispersal, and provides further information on the life history of this species.

MATERIALS AND METHODS

Location

The population of *C. convexa* studied lives on a sand flat adjacent to the New Jersey Oyster Research Laboratory near Green Creek, lower Delaware Bay (New Jersey). The habitat consists of a broad, very gently sloping tidal flat that extends bayward from a narrow, marsh-bordered beach. Water temperature varies seasonally from near freezing to 30° C and may fluctuate as much as 5° C during a summer

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[1] A contribution from the New Jersey Oyster Research Laboratory, Rutgers—The State University.

tidal cycle. Salinity also varies seasonally but usually remains within the range of 20–26‰, although a change of up to 2‰ during a single tidal cycle is not uncommon. In addition to temperature and salinity stresses, epibenthic organisms in this habitat are subjected to dehydration, severe siltation and burial, and winter ice scouring.

Massive quantities of clam shells (*Spisula solidissima*) are distributed near shore each July as cultch (artificial substrate) for metamorphosing oyster larvae. During autumn most cultch and attached oysters are transplanted to deeper waters while the remainder may become assimilated into a small oyster reef. *Crepidula convexa* appears each summer on newly distributed cultch and a portion of the population remains on the reef each autumn and through the winter.

Methods

For almost 2 years monthly collections were made during low tides. Specimens on cultch and others on *Pagurus* (Hermit Crab)-inhabited snail shells were gathered separately. Those not immediately examined were held in running baywater in the laboratory. Every specimen of *C. convexa* on each piece of substrate was detached and inspected foot-up in a dish of baywater. Sex was determined by the stage of development of the phallus in relation to the body size of the living animals, and shell length was measured with a calibrated stereomicroscope.

C. convexa passes through five recognized sexual phases: immature; phallus-bud; male; transitional; female. Immature animals exhibit only a slight prominence on the right side of the neck, at the site of the developing phallus. Phallus-bud snails possess a small and peglike phallus. Males are animals with a muscular, functional phallus. Transitional phase animals, produced from males and possibly also from phallus-buds, are characterized by a degenerate phallus, small in relation to total body length. Females are the largest animals and frequently retain a diminutive remnant of the phallus.

RESULTS

The intertidal *C. convexa* is primarily found in cultch areas that remain wet during low tide or on *Nassarius obsoletus* shells inhabited by *Pagurus longicarpus*. In the remainder of this report, *Crepidula* on cultch is referred to as "cultch forms" and those on the *Nassarius* shells as "*Pagurus* forms."

Fecundity

The data on the fecundity of Delaware Bay *C. convexa* presented below are based on 250 brooding female-phase snails examined during the summer of 1968.

The egg mass of *C. convexa* is similar in form to that of other Calyptraeidae. Each is composed of a group of egg capsules joined by a sticky pad (Fig. 1). During an incubation period lasting approximately two weeks, the egg mass is held beneath the ventral face of the neck lappets. It may either be cemented to the substrate by the sticky pad or the pad may be attached to the propodium and thus unattached to the substrate.

The egg capsules have a feature not described for other members of the genus. Each is divided into two compartments (Fig. 1) that hold approximately equal numbers of eggs. The capsule is initially folded in half along the axis of the stalk,

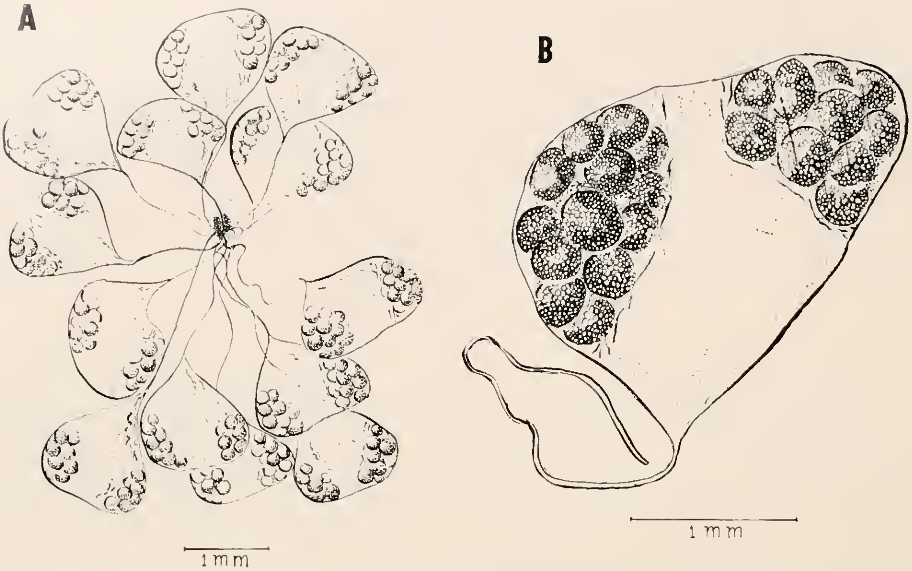

Figure 1. Egg mass and egg capsules of *C. convexa;* (A), an egg mass, consisting of a series of capsules radiating from a sticky tab which may be attached to the substrate; (B), a single capsule showing ova separated into two compartments, drawn from preserved sample flattened under a coverslip.

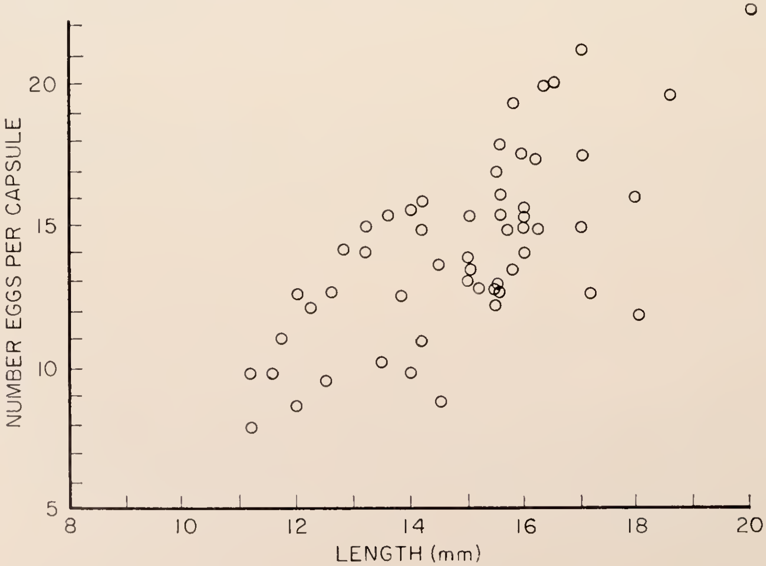

Figure 2. Relationship between shell length and the number of eggs per capsule; cultch form, June, 1968.

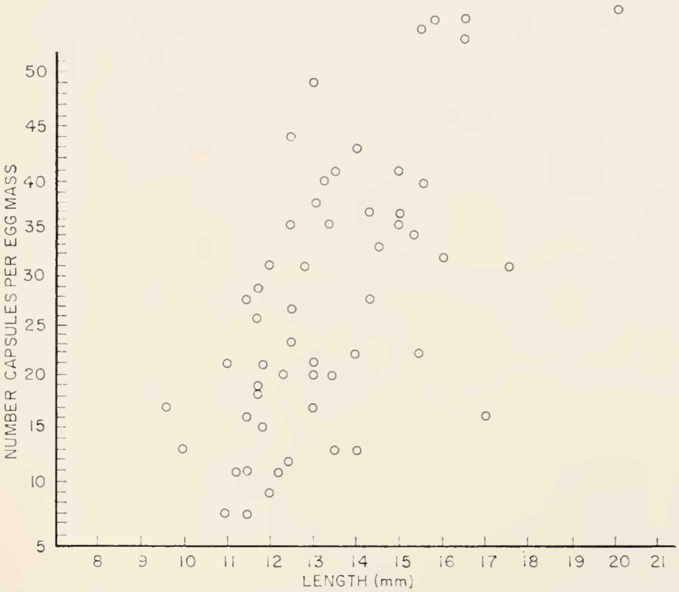

FIGURE 3. Relationship between shell length and the number of egg capsules per egg mass; cultch form, June and July, 1968.

but unfolds to accommodate the growing embryos. The compartment membranes disappear by the time the embryos have developed to a motile stage.

For the purposes of the following discussion, four stages in larval development are recognized: *early gastrula,* including all stages from the zygote to the appearance of external cilia; *trochophore,* for the ciliated motile larva prior to the appearance of the velum; *veliger,* for both shelled and shell-less embryos that possess a velum; and *post-veliger,* for larvae in which the velum is resorbed and the foot is well developed.

Table I shows the occurrence of each of these developmental stages per capsule during June and July, 1968. Each observation represents the average of five capsules from a single egg mass (all of the capsules in a given egg mass are at approximately the same stage of development). These data reveal that there is a significant decline in the numbers of veligers per capsule in relation to trochophores, from an average of about 14 per capsule to 11 per capsule.

The relationship between shell length and both the numbers of eggs per capsule and the number of capsules per egg mass is shown in Figures 2 and 3 (cultch form only). Although the variability is high, it is evident that both of these aspects of fecundity are size-specific, the largest animals producing the greatest numbers of eggs per capsule as well as more capsules per egg mass. These figures also indicate that egg production begins in the size range of 10–11 mm although, infrequently, smaller female-phase snails may oviposit.

Total fecundity expressed as the total number of eggs per mass, and as the total number of surviving veligers and post-veligers, is shown in Figure 4 as a function

TABLE I

Occurrence of various developmental stages per egg capsule
(Data includes both cultch and Pagurus *subpopulations, 1968)*

	No. animals	Mean eggs/capsule	No. animals	Mean trochophores/capsule	No. animals	Mean veligers/capsule	No. animals	Mean Post-veligers/capsule
June	68	13.9	25	14.4	14	11.8	18	9.8
July	25	14.2	4	15.5	17	10.7	28	11.5
$\overline{\overline{X}}$		13.8		14.6		11.2		10.9

$\overline{\overline{X}}$ Eggs = $\overline{\overline{X}}$ Trochophores > $\overline{\overline{X}}$ Veligers = $\overline{\overline{X}}$ Post-Veligers (at $P < 0.05$).

of animal size. Egg production varies from somewhat less than 200 per mass to a maximum in excess of 1300 per mass. However, the numbers of embryos reaching the veliger and post-veliger stages range from less than 100 per mass in small animals to close to 1000 in large females. The reduction in the numbers of embryos which occurs during development (Table I) is therefore a phenomenon which clearly exists over the entire size range of ovipositing females.

It is likely that the abortion and disintegration of the lost embryos provides a nutrient source for the surviving embryos. This form of "embryonic cannibalism" was reported also by Coe (1942a) in *Crepidula onyx,* and by Thorson (1940) in *C. walshi.* Thus, it would seem that the contention of Fretter and Graham (1962, page 404) that this is not a normal occurrence in *Crepidula* is not supported. It seems quite extraordinary to us that a direct-developing species like *C. convexa* would, as a matter of course, uselessly expend energy by incorporating more eggs per capsule than will develop.

The fecundity data represented in Figure 4 above reflects egg production per egg mass. It is probable that two or perhaps even three broods may be produced during a summer. We have observed that animals brooding eggs will produce a second egg mass when the eggs are removed. The maximum percentage of brooding females occurs in June and declines in July. Thus egg production is concentrated in a period of about two months between late May and late July. Since the incubation period requires about two weeks, the average production of about three broods per season is further confirmed.

Table II summarizes the fecundity data for the Delaware Bay population and compares it with information provided by Coe (1949) for a Massachusetts population from Woods Hole. The differences are remarkable. Egg diameter is larger, as is the number of capsules per mass and the total number of eggs per mass in the southern population. These differences may reflect not only the longer growing season in Delaware Bay but also the very productive environment there, conducive to the rapid growth of filter-feeding invertebrates such as *Crassostrea virginica* and *Crepidula.*

Life history

Post-veliger juveniles crawl inside the capsule prior to hatching and may remain in a tight clump under the female for more than a day after hatching. *Crepidula*

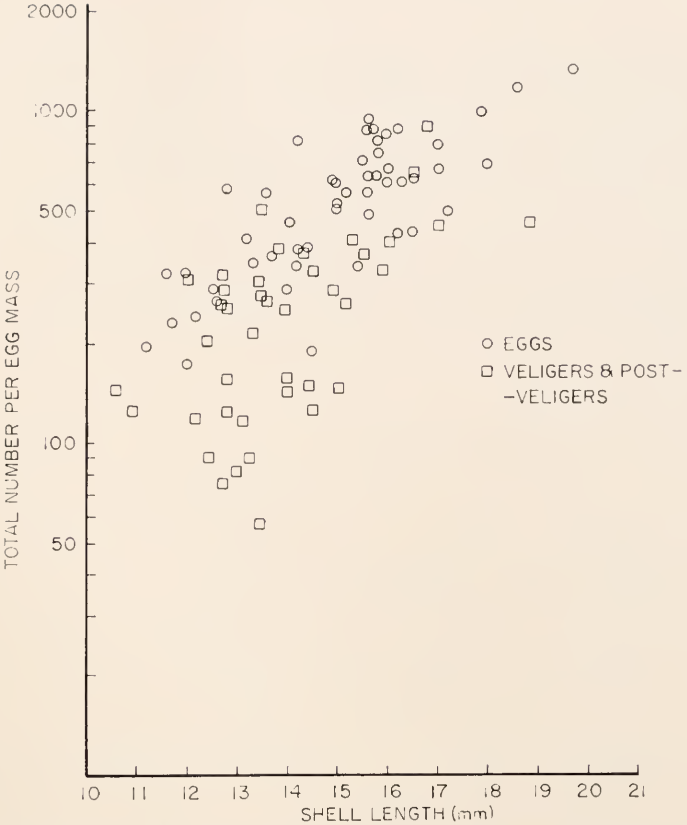

FIGURE 4. Relationship between shell length and: (1) total number of eggs per mass and (2) total numbers of veligers and post-veligers per mass; June and July, 1968.

convexa was not observed to lift its shell and eject its young as does *C. adunca* (Putnam, 1964). In emergent juveniles, 0.95 ± 0.09 mm long, the shell apex is posterior and medial. By the time the shell is 1.5 mm, radial purple stripes appear.

An individual may pass through all sexual phases in its first summer, and size ranges for the successive sexual phases overlap. The immature and phallus-bud stages are respectively 1–8 and 2–9 mm. The males are 1–10 mm, although most individuals with a well-developed phallus are at least 4 mm. The transitional

TABLE II

Fecundity of Crepidula convexa—*Woods Hole and Delaware Bay*

Location	Egg diameter (microns)	No. eggs per capsule	No. capsules per egg mass	Total eggs per egg mass	Reference
Woods Hole	280	8–20	15–25	250 (maximum)	Coe (1949)
Delaware Bay	320*	12*	33*	442*	Present paper

* Mean values based on 250 egg masses, summer 1968.

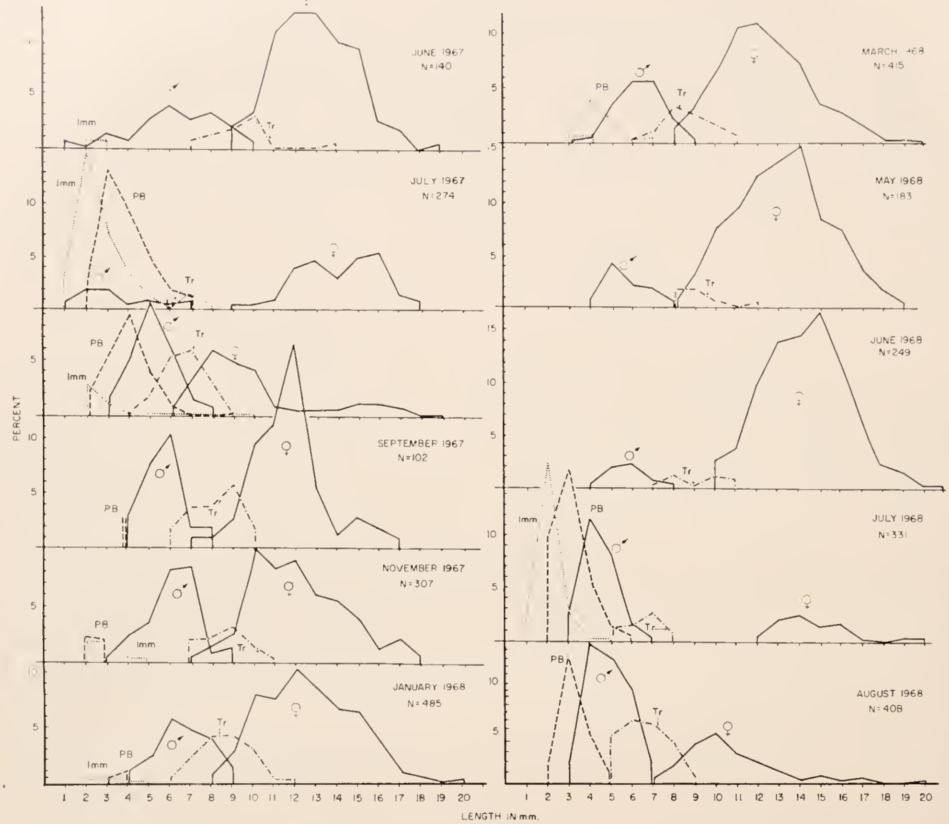

FIGURE 5. Size-frequency histograms for the cultch subpopulation; June 1967–August, 1968.

forms range from 5–14 mm, overlapping both phallus-buds and males. The existence of transitional forms, with the phallus-length to body-length ratio of phallus-buds and body length of large males, suggests that the male phase may be omitted in nature as well as in experimental isolation. The females range from 6–20 mm and overlap all other sexual stages in size. Transition to the female phase can definitely be accomplished in one season, since 7.0 mm females produced eggs during their first summer. The occurrence of females as large as 20.0 mm suggests a life span of two seasons ($1\frac{1}{2}$ years) or more.

Crepidula convexa does not form the permanent chains of mated animals characteristic of some species in the genus. At any one time, a female carries only one attached male. If a third individual is attached to the mated pair, it is generally sexually immature.

Data summarizing the sexual composition of the cultch subpopulation are shown in Figure 5; of the *Pagurus* subpopulation in Figure 6. Figures and present data on the per cent of females brooding egg masses and of females associated with male-phase animals.

Annual population cycle

Oviposition probably begins in late April or early May coincident with the seasonal increase in water temperature. Eggs were first observed in the field on 12 May, when water temperature had risen to about 15.0° C. At this time, females dominate the population (Fig. 5) and the percentage of brooding females approaches 100 per cent. This percentage declines to about 50 per cent in July, and to 25–30 per cent in August. Factors mediating this decline may be depletion of sperm in the seminal receptables, exhaustion of energy reserves, and an increasing percentage and number of newly transformed females which have not yet been inseminated.

Brooding of the new year class in June results in a large number of immature and phallus-bud individuals in July. These, plus older males, comprise the pop-

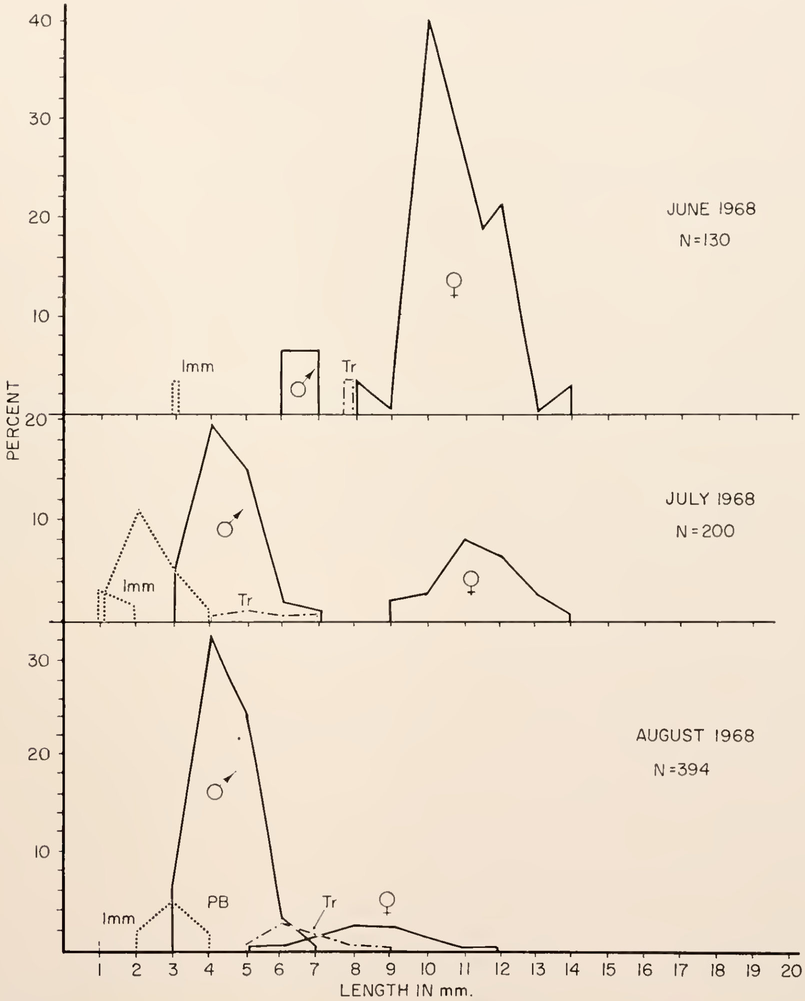

FIGURE 6. Size-frequency histograms for the *Pagurus* subpopulation; June–August, 1968.

ulation components at the left of the July histograms (Fig. 5). The component on the right consists only of older females.

During August the immatures and phallus-buds which appeared the previous month transform to males. Consequently, a large component of males is generated at the left on the August histogram and the number of immatures declines. The same histograms show a simultaneous increase in female and especially in transitional stages. The flattening at the right end of the histogram, however, indicates that a large proportion of the females are recently transformed and therefore of smaller size. These transitions and females have been produced from males of the previous year, and perhaps to a lesser extent from males of the new year class.

Throughout June, July and August, the number of mated males (males associated with females in a mating position) increases from less than 10 per cent to 30–40 per cent, in concert with decreasing oviposition. Since the ratio of males to females is approximately constant for both June and July, the increase in mating at this time must result from an increased tendency for males to mate. In August, the influx of recently transformed males further increases mating. By September, males and females are the two principal sexual components of the population although the young stages present in August remain important. The transitional phase now encompasses an increased size range, indicating that larger males are becoming transitional and larger transitional phases becoming females. At the same time, most of the immatures are transformed to males. This September distribution pattern is in sharp contrast with the nearly constant ratio of male to transitional phases in July and August. In September, males are animals spawned during the summer while females include animals spawned in both the current and the previous summer.

The percentage of large females increases from September to November. Growth is also shown by the progressively greater modes of the female and other phases from November to March. The similarity of the histograms for the winter months and the accumulation of female phases indicates a slow but significant transformation of sexes during this period. Consequently females (many of them recently transitional unmated) dominate the population in May and the cycle starting with spring oviposition begins again.

Cultch and Pagurus *subpopulations—The phenomenon of dwarfism*

Studies of the dual substrate forms of *Crepidula convexa* (Franz and Hendler, 1969) have shown that the broad, flat shell shape of the cultch form and the narrow, high shape of the *Pagurus* form is caused by limitations inherent in the available substrate. Shell growth in *Pagurus* forms is restricted by the length and curvature of the *Nassarius* shell. Hence large *Pagurus* forms tend to orient on the long axis of the *Nassarius* shell, with their head at the aperture; they often produce a skirt of shell which overgrows the sides of the *Nassarius* shell. Because of substrate restrictions *Pagurus* form females attain a maximum length of only 14.0 mm while cultch forms of almost 20.0 mm are present throughout the year.

In addition to size and shape, the percentage of females with attached males in the *Pagurus* subpopulation (up to 80%) exceeds the cultch subpopulation (5–30%). This probably reflects the lack of space and the isolation of *Pagurus* forms on moving hermit crabs. The more consistant association of male and female *Pagurus*

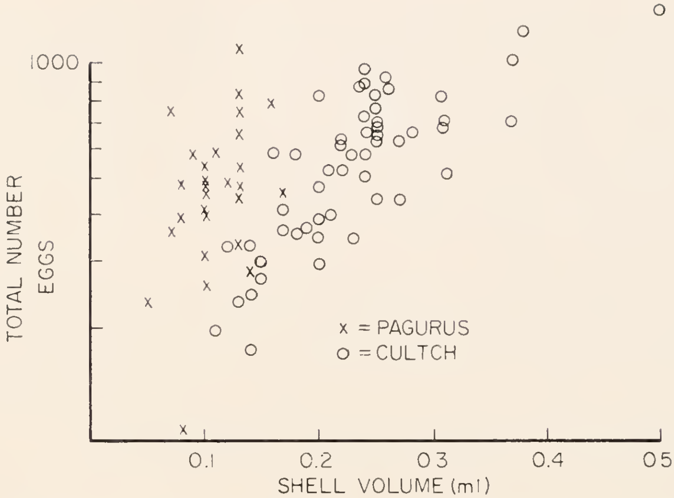

FIGURE 7. Relationship between the total number of eggs per egg mass and the shell volume of *Pagurus* and cultch subpopulations.

forms may account for the longer oviposition period as compared with the cultch forms.

The two substrate subpopulations differ also in their tendencies to attach the egg mass to the substrate. An average of 20 per cent of cultch animals but 70 per cent of the *Pagurus* animals cement egg masses to the substrate rather than clasping them beneath the foot. These differences between the subpopulations suggest that attachment is affected by some environmental stimulus.

Figure 7 shows an additional contrast between the substrate-controlled subpopulations. Although the maximum shell volumes of cultch and *Pagurus* forms are similar (Franz and Hendler, 1970), the shells of most of the ovipositing *Pagurus* forms are less than 0.15 ml, whereas ovipositing cultch forms generally exceed this volume. As mentioned previously, the fecundity of the cultch forms is a size function, females of greater volume producing more eggs. The relationship between female shell volume and fecundity is less clear for the *Pagurus* forms because a majority of the brooding females are less than 0.15 ml volume, while a large number of brooding cultch females attain at least 0.3 ml. It is noteworthy that the total fecundity range for the two forms is the same, the number of eggs per female varying between roughly 100 and 1000. However, *Pagurus* females produce more eggs than cultch females of equal volume, a difference probably indicating a different degree of sexual maturity at an age when their volumes are similar. In other words, large *Pagurus* forms are almost certainly older than cultch forms of equal volume. A growing *Pagurus* form female transferred to a flat substrate would therefore be expected to produce the same number of eggs as a cultch form of greater volume. It appears then that *Crepidula convexa* living in association with *Pagurus* constitutes a true dwarf population, but without any indications of the neoteny observed by Thorson (1965) for *Capulus* associated with *Turritella.*

Changes in the sexual composition of the *Pagurus* subpopulation analogous to those in the cultch subpopulation occur during the summer (Fig. 6) but transformations may be more rapid in the *Pagurus* form. Both transitional and juvenile stages are relatively rare, possibly because they exist for only short periods and therefore are not adequately represented in the sample. More rapid transformation may also result in exaggerated percentages of *Pagurus* form females in June as of males in August compared to the cultch form sex ratio at these same times. These differences between the subpopulations could be either a direct effect of the substrate or perhaps the indirect result of the greater amount of mating association among the *Pagurus* animals.

Differences between the substrate forms in morphology, mating behavior, breeding cycle, and maturation rate are attributed, directly or indirectly, to the substrate. The *Pagurus* and cultch form populations are not, however, autonomous. There is an interchange of individuals from different substrates mediated by phoresis on *Pagurus*. This exchange prevents the isolation of the two substrate forms and permits *Pagurus* forms to escape crowded hermit crab shells.

Movement of *Pagurus* forms to cultch is suggested by the appearance of male *C. convexa* on bare, isolated surfaces such as newly deposited cultch mounds. These mature settlers do not grow from juveniles present on the surface, nor are they spawned by females in the vicinity; neither do they move by themselves across sand and mud sediments to the cultch mounds.

The feasibility of phoresis was tested in the laboratory. Within 30 hours, *C. convexa* moved both ways, from *Pagurus* to clean cultch, and from cultch to unoccupied *Pagurus*. The animals that transferred to alternate substrates in both tanks were small (4.4 ± 1.4 mm), and were predominantly immatures, males, and few transitional phases. A similar experiment on the sand flat indicated that phoresis is operative in the field. Thus, *Pagurus* in the vicinity with mobile, young *Crepidula* can serve as vectors in the colonization of virgin substrates.

Discussion

In view of the success of congeneric planktotrophic species such as *Crepidula plana* and *C. fornicata,* the existence of direct development in *C. convexa* is interesting, and raises questions as to its adaptive advantage, and its ecological significance. Direct development is generally thought to be an adaptation to extreme environmental stresses such as occur in estuarine, arctic or deep-sea situations (Thorson, 1950). The advantages conferred by direct development include: decreased larval mortality from predation, independence from a planktonic food source, and ready access to suitable substrate. The chief liabilities are the loss of a larval dispersal mechanism and reduction in numbers of young. Assuming that dispersal is desirable, only organisms with strong inherent mobility would be expected to evolve direct development.

The results of this study on the sexual dynamics of the population shed light on the means by which *C. convexa* has circumvented or neutralized the liabilities noted above. Young animals are highly mobile and adept at phoresis, thus facilitating dispersal in the absence of a delicate veliger larva. Moreover, the high mobility of males permits promiscuity so that males mate with more than one female during the breeding season, thereby increasing the reproductive capacity of the population.

The high mobility of young *C. convexa,* affecting as it does both the capacity for dispersal and reproductive success, accounts for the abilities of the species in colonizing new substrates as well as the expansion of its geographic range as noted by Vokes (1935).

Since direct development in *C. convexa* appears linked to a substitute mechanism for larval dispersal, it is possible that a mating system involving mobile males preceded the evolution of direct development. We do not suggest that this evolutionary sequence is a necessary prerequisite for the evolution of direct development in other *Crepidula* species. We feel, however, that in most cases the evolution of direct development involves a complex of adaptations which may be, but are not necessarily, induced by extreme environmental stress. The ultimate problem is to ascertain the actual conditions that initiated the production of direct development from planktotrophic forms and to determine how the eventual sympatry of these forms has come about.

Additional comparative studies on similar forms with direct and planktotropic development are needed before the progressive stages in the evolution of direct development can be uncovered. An ideal situation for such a study exists on the Pacific coast of the United States where, according to Coe (1949) up to 6 *Crepidula* species may coexist, 3 with planktotrophic larvae, and 3 with direct development.

This research was performed at the New Jersey Oyster Research Laboratory, Rutgers—The State University. We gratefully acknowledge the aid, assistance, and encouragement of Dr. Harold H. Haskin, Rutgers University, and the help of the Oyster Research Laboratory staff, especially Mr. Walter Canzonier.

SUMMARY

1. The reproductive biology and population dynamics of two substrate forms *Crepidula convexa* were investigated. The species is a protandric hemaphrodite with direct development.

2. The egg mass differs from other species in the genus in that the capsules comprising it are compartmentalized in early development. Later, the compartment walls break down and there is a decrease in the number of embryos, suggesting the existence of "embryonic cannibalism." Size-specific fecundity of the Delaware Bay population appears higher than published data for a Woods Hole population.

3. There is rarely more than one male associated with a female and the ratio of males to females is low.

4. Life span is at least two seasons. At the beginning of June, when reproduction is maximal, the population is predominantly female. The resulting influx of juveniles shifts the sexual composition of the population in such a way that males dominate by August. The transformation of these males to females during the winter gradually shifts the sexual composition so that females again predominate by Spring.

5. *Crepidula convexa* living on *Pagurus* form a dwarf population which differs from cultch animals in morphology, mating behavior, breeding cycle and maturation rate. These differences are attributed to limitations of substrate. Exchange of individuals between subpopulations is facilitated by phoresis on hermit crabs.

6. The high mobility of young *C. convexa*, which makes phoresis possible, is considered to be the key factor in the success of this species in the colonization of virgin substrates, thus facilitating dispersal in the absence of a larval stage. The capacity of males to fertilize more than one female is also a function of this mobility and is probably the major factor in maintaining a sex ratio (males < females) which favors maximal reproduction.

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IN VITRO DEVELOPMENT OF INSECT TISSUES. I. A MACROMOLECULAR FACTOR PREREQUISITE FOR SILKWORM SPERMATOGENESIS [1]

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Blood of metamorphosing silkworms contains a "macromolecular factor" (MF) which is indispensable for the maturation of their spermatozoa. This fact was documented nearly twenty years ago (Schmidt and Williams, 1953) in an investigation in which germinal cysts containing primary spermatocytes were removed from the testes of diapausing Cecropia or Cynthia pupae and cultured in hanging drops of hemolymph. In the absence of MF, the spermatocytes survived but failed to develop. By contrast, when MF was present in the culture medium, meiosis began within 24 hours and was followed by the rapid differentiation of spermatids and spermatozoa within the elongating germinal cysts. MF proved to be an undialyzable, non–species-specific factor which was stable at 75° C but rapidly inactivated at higher temperatures.

In assays of hemolymph removed from male or female Cecropia silkworms at successive stages in metamorphosis, Schmidt and Williams (1953) detected large and systematic changes in the titer of MF. For example, little or no activity was encountered in the blood of fifth instar larvae or of diapausing pupae. Substantial activity was detected during and immediately after pupation and, months later, during the termination of diapause and initiation of adult development.

Since virtually all aspects of insect metamorphosis including *in vivo* spermatogenesis were known to involve a hormone secreted by the prothoracic glands, Schmidt and Williams (1953) suggested that MF might constitute that hormone. This suggestion became untenable a year later when Butenandt and Karlson (1954) isolated ecdysone—a heat-stable, dialyzable steroid which, on injection into immature insects, provoked all the *in vivo* developmental phenomena previously realized by the implantation of living active prothoracic glands. Among these phenomena was the swift initiation of spermatogenesis when diapausing saturniid pupae were injected with ecdysone.

Yet, strange to say, ecdysone when added to the culture of germinal cysts proved completely ineffective in provoking *in vitro* spermatogenesis or in substituting for MF (C. M. Williams, as quoted by Karlson, 1956, page 248). Moreover, in unpublished experiments which Williams carried out in collaboration with Karlson, no evidence could be found for a complexing of ecdysone with any MF-like protein.

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For these several reasons, MF became and has remained something of an enigma.

During the past three years we have undertaken a detailed reexamination of *in vitro* spermatogenesis with special reference to the role of MF. In the present report we describe the production of MF, its changing concentration in the hemolymph of diapausing pupae, and its extraction and partial purification.

Materials and Methods

1. *Experimental animals*

Most experiments were performed on diapausing pupae of *Samia cynthia* derived from larvae reared on ailanthus trees under short-day conditions. The cocoons containing the pupae were stored at 25° C under which condition the diapause persists indefinitely. A few experiments were performed on three other species—namely, *Antheraea polyphemus*. *A. mylitta*, and *Hyalophora cecropia*. These species were reared or purchased from dealers.

2. *Preparation and evaluation of tissue cultures*

The technique of Schmidt and Williams (1953) was adapted with minor modifications. Groups of No. 1 coverslips wrapped in aluminum foil were heated overnight at 140° C. All other glassware was boiled in aqueous detergent ("Alconox," Alconox, Inc., New York) for 20 minutes, rinsed several times in tap water and distilled water, submerged for 10 minutes in 70% ethanol, and heat-dried at 140° C. Each item was then individually wrapped in foil and again heat-sterilized at 140° C overnight. Metallic instruments were soaked in 70% ethanol for 20 minutes and wiped dry with sterile tissue.

The pupae were surface-sterilized by submerging them for two minutes in 0.05% mercuric chloride in 50% ethanol, followed by several rinses in sterile distilled water. To collect samples of blood, a V-shaped incision was made at the tip of one or both forewings and the hemolymph was expressed into a chilled, sterile centrifuge tube containing a few crystals of phenylthiourea (PTU) to inhibit tyrosinase activity. The blood was centrifuged at 4° C for 15 minutes at 17,600 g and decanted into a sterile tube containing a few crystals of PTU. Hemolymph treated in this manner will be referred to as "plasma" in contradistinction to uncentrifuged "whole blood" containing hemocytes.

Pairs of testes were dissected from male pupae, placed in sterile disposable Petri dishes, separated from fragments of attached fat body, and rinsed in sterile insect culture medium (Grace, 1962). They were transferred to a second sterile dish, again rinsed in Grace's medium and teased open in a depression slide containing 200 μl of the desired culture medium. The testicular walls were discarded and the suspension of germinal cysts was subdivided onto six sterile coverslips. The latter were inverted and sealed with melted wax above the concavities of depression slides. Each culture contained 50–100 cysts in the volume of 30–45 μl medium.

The cultures were immediately examined under a compound microscope at 100 ×. As a rule they contained cysts arrested at the pachytene stage of the first meiotic division; a small percentage was at the earlier spermatogonial stage. In the rare instances where any cysts contained spermatids, the preparations were discarded.

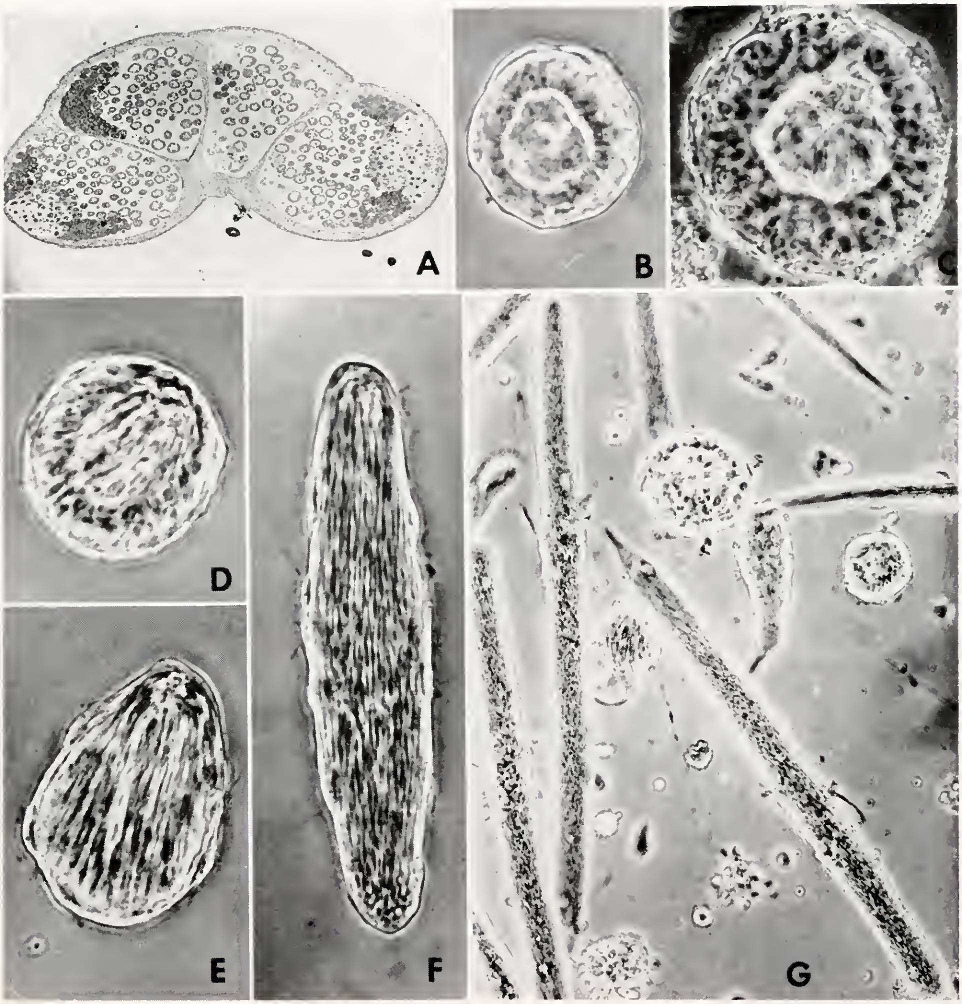

FIGURE 1. With the exception of Figure 1A (which is a section of a fixed and stained preparation), all other half-tones here and in Figure 4 are of phase contrast photographs of living cultures. (A.) Section of the testis of a diapausing Cynthia pupa; the germinal cysts occupy the four chambers formed by the inflections of the inner layer of the testicular walls (40 ×). (B.) A typical germinal cyst removed from a diapausing Cynthia testis. The primary spermatocytes surround the central lumen and are enveloped in a thin layer of follicle cells; Stage I according to the terminology of Schmidt and Williams (1953) (470 ×). (C and D.) Cysts after 24 hours of culture in MF-containing medium, meiosis has been completed and the axial filaments are oriented to the center of the lumen, Stage II (C = 660 ×; D = 470 ×). (E.) The spermatids and the cyst as a whole have begun to elongate, Stage III (470 ×). (F.) The cyst is now more than twice as long as wide, Stage IV (490 ×). (G.) Typical appearance of cultures after 7 days in MF-containing media. The very elongate cysts contain bundles of fully developed sperm. The less elongate were formed from cysts containing spermatogonia when the culture was prepared (160 ×).

The cultures were placed in a dark incubator at 25° C. Every 24 hours for a period of 15 days, they were reexamined. The cysts in each culture were counted and their developmental condition scored according to the classification of Schmidt and Williams (1953) (Fig. 1). The scoring was repeated for each culture and the average number of cysts in each stage was calculated each day. Cysts in developmental stages III and IV (E and F in Fig. 1) were scored as "positive"; these were summed and used to calculate the percentage of developing cysts which was used as a measure of MF titer. This calculation was ordinarily made on the tenth day.

3. *Culture media*

Most cultures were prepared in PTU-treated plasma or whole blood obtained from male or female pupae at specific stages in development. Certain cultures were prepared in "Grace's insect TC medium without insect hemolymph" (Grand Island Biological Co., Cat. No. 159). One series of experiments made use of three mammalian blood sera purchased from G.I.B. Co.—namely, Cat. No. 617, Calf serum; Cat. No. 614, Fetal calf serum; and Cat. No. 601, Newborn calf serum.

Undiluted insect blood or plasma is resistant to infection and it was not necessary to take any additional sterile precautions. However, when other media were utilized, the cultures were prepared in a UV-sterilized, plastic glove-box.

Osmolarities were estimated by the freezing-point method using a Model G-62 Fiske osmometer.

RESULTS

1. *MF activity in the plasma of diapausing Cynthia pupae*

Newly spun cocoons of the Cynthia silkworms were stored at 25° C under 12 hours of daily illumination. At successive intervals 5 to 10 pupae were removed from their cocoons and bled. Each sample of blood was centrifuged and the undiluted plasma used to prepare a minimum of 30 hanging-drop cultures of germinal cysts derived from the testes of diapausing Cynthia pupae. The developmental responses were scored as described under METHODS and averaged for each group of cultures.

The results summarized in Figure 2 reveal large and systematic changes in MF activity in the plasma of both male and female pupae. The curve shows three self-evident phases: (1) a rapid decline during the first 8 weeks after pupation; (2) zero activity from the 8th to the 10th week; and (3) a reappearance of activity on the 11th week and a progressively increasing titer thereafter. The experiment was twice repeated with the same results.

These large changes in MF are of special interest because they take place in pupae which show no trace of the termination of diapause. In this sense the present results are not in full accord with those reported by Schmidt and Williams (1953).

2. *Inability of ecdysone to substitute for MF*

Twenty-one cultures of germinal cysts were prepared in the plasma obtained from diapausing Cynthia pupae after 2 to 3 months at 25° C. To twelve of these cultures α- or β-ecdysone was added in a final concentration of 1 μg per 100 μl

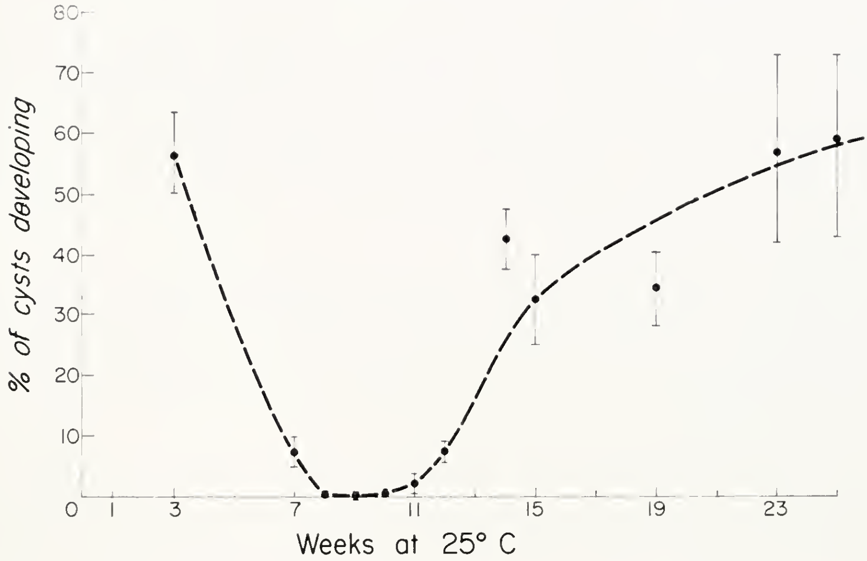

FIGURE 2. Changes in MF activity in the blood plasma of uninjured diapausing Cynthia pupae stored at 25° C. The vertical lines indicate the range of the measurements.

medium. The results as summarized in Table I reveal that the low but finite MF activity of the blood was not enhanced by the addition of ecdysone. The experiment was repeated on the plasma of pupae previously stored at 25° C for 4 to 5 months. Here again, the presence of ecdysone was inconsequential.

In an additional experiment 40 cultures were prepared in Grace's medium. In this case some of the cysts survived for 3 or 4 days but showed no trace of development in either the presence or absence of ecdysone. The rest of the cysts showed progressive dissociation into free spermatocytes and follicle cells all of which soon died.

These findings therefore confirm the previously cited conclusion that ecdysone is inactive in the *in vitro* spermatocyte assay and cannot substitute for MF.

TABLE I

Culture of Cynthia cysts in blood plasma of diapausing Cynthia pupae or in Grace's medium; ineffectiveness of ecdysone in provoking spermatogenesis

Culture media	Ecdysone not added		Ecdysone* added	
	No. cultures	% cysts developing**	No. cultures	% cysts developing**
Blood plasma:				
From pupae 2 to 3 months at 25° C	9	8 ± 4	12	3 ± 1
From pupat 4 to 5 months at 25° C	9	25 ± 6	12	26 ± 4
Grace's medium	20	0	20	0

* Either α- or β-ecdysone added in final concentration of 1 μg per 100 μl medium.

** Here and in subsequent Tables the mean and its standard error are recorded.

3. *In vivo activation of the plasma*

In an unpublished study which he carried out as a Harvard undergraduate, Dr. Robert D. Yee, of the University of Rochester School of Medicine, found that high titers of MF were routinely encountered in the blood of diapausing pupae after integumentary injury. We have confirmed Yee's important finding in the extensive series of experiments summarized in Table II. In these experiments diapausing pupae of three different genera were stored at 25° C for specific periods and then treated in the following manner:

An initial sample of blood (*ca.* 200 μl) was collected from each pupa. The sample was centrifuged and assayed for MF in order to determine the initial activity prior to injury. Each pupa was anesthetized with carbon dioxide for 25 minutes; it then received a large integumentary injury consisting of the excision of the tip of the abdomen which was sealed with a plastic window (Williams, 1959). After storage at 25° C for 24 hours the blood of each pupa was collected, centrifuged, and the resulting plasma assayed in the usual way.

The results summarized in Table II reveal a spectacular increase in MF activity in response to integumentary injury. Particularly impressive is the result obtained on the Cynthia pupae whose plasma showed zero MF prior to injury and a titer of 43% after injury.

4. *Dynamics of injury response; effects of hemocytes*

To clarify the time-course of the MF response to injury, the following experiment was carried out on a homogeneous group of 18 diapausing Cecropia pupae. Two hundred μl of hemolymph were first collected from each individual. Each

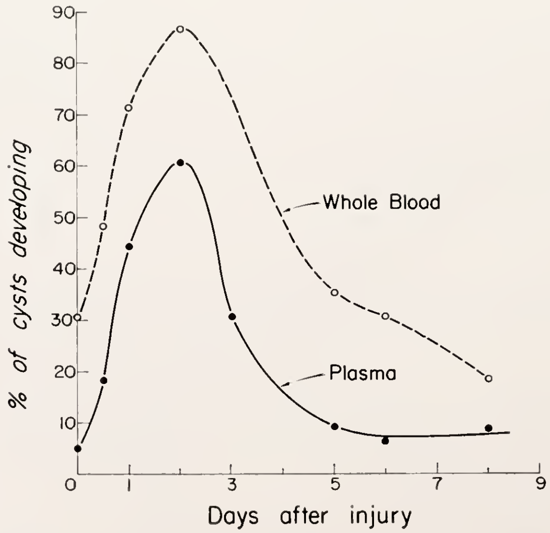

FIGURE 3. Changes in MF activity in the hemolymph of diapausing Cecropia pupae which were injured and then stored at 25° C. The upper curve is for assays on uncentrifuged blood; the lower curve is for blood plasma lacking any blood cells.

then received a large integumentary injury consisting of the excision of the tip of the abdomen, the wound being sealed by a plastic window. Two individuals were immediately sacrificed and bled. The remainder were stored at 25° C; at predetermined intervals two additional pupae were sacrificed and bled. All samples of blood including the initial samples were subdivided into two aliquots, one of which was centrifuged to remove the blood cells. The MF activities of all samples were assayed in the usual way on germinal cysts of Cynthia. Altogether, a total of 93 hanging-drop cultures were prepared from plasma and 89 from whole blood.

As indicated in the lower curve of Figure 3, the activity of the plasma was uniformly low in all pupae prior to injury (average 5%; range 0 to 8%). Within 12 hours after injury the plasma already showed a substantial increase in MF. The activity became maximal on the second day; it then underwent exponential decay during the following four days to the levels approximating those prior to injury. The upper curve in Figure 3 summarizes equivalent data for cultures prepared in uncentrifuged blood. Even the initial samples showed substantial MF activity (30 ± 6%). Here again, the activity was maximal in pupae sacrificed two days after injury.

In many additional experiments performed on Cecropia and Cynthia we have routinely found substantially greater MF activity in cultures containing whole blood rather than plasma. Evidently, the living hemocytes are able to contribute MF to the cultures over and above that already present in "injured" plasma. A direct test of this hypothesis was carried out in the experiment that follows.

5. *MF from cultured hemocytes*

Sixteen diapausing Cynthia pupae were utilized 7 months after pupation. Each individual received a large integumentary injury (excision of the tip of the abdomen and resealing with a plastic window). Seventy-two hours later the pupae were maximally bled into chilled centrifuge tubes containing a few crystals of an equal part mixture PTU and streptomycin sulfate. The hemocytes were collected by centrifugation (5 minutes at 3000 *g*). The plasma was discarded and the cells were washed three times in successive volumes of Grace's medium (total of 10 ml). Each of the 16 preparations of hemocytes was then resuspended in 2.5 ml of Grace's medium and placed in a 30 ml plastic culture flask (Falcon) at 25° C.

Within the first hour the hemocytes adhered to the plastic; they began to proliferate and within 72 hours had coated the plastic surface as a monolayer. This impressive multiplication (Fig. 4A–C) is of special interest since these blood cells constitute the only cell type which we have been able successfully to culture in Grace's medium unfortified with plasma or other macromolecules. This fact in itself suggests that the hemocytes are able to condition the medium by releasing macromolecules into it.

In the experiment in question the germinal cysts from two testes of Cynthia pupae were introduced into each culture after 24 hours. In 15 of the 16 cultures many of the cysts underwent meiosis and the initiation of spermatogenesis within one to two days. After three days, 10 to 70% of the cysts showed the positive assay for MF.

This clear-cut response was therefore impressively different from the absence of development and, in fact, the death within 3 to 4 days of germinal cysts cultured in

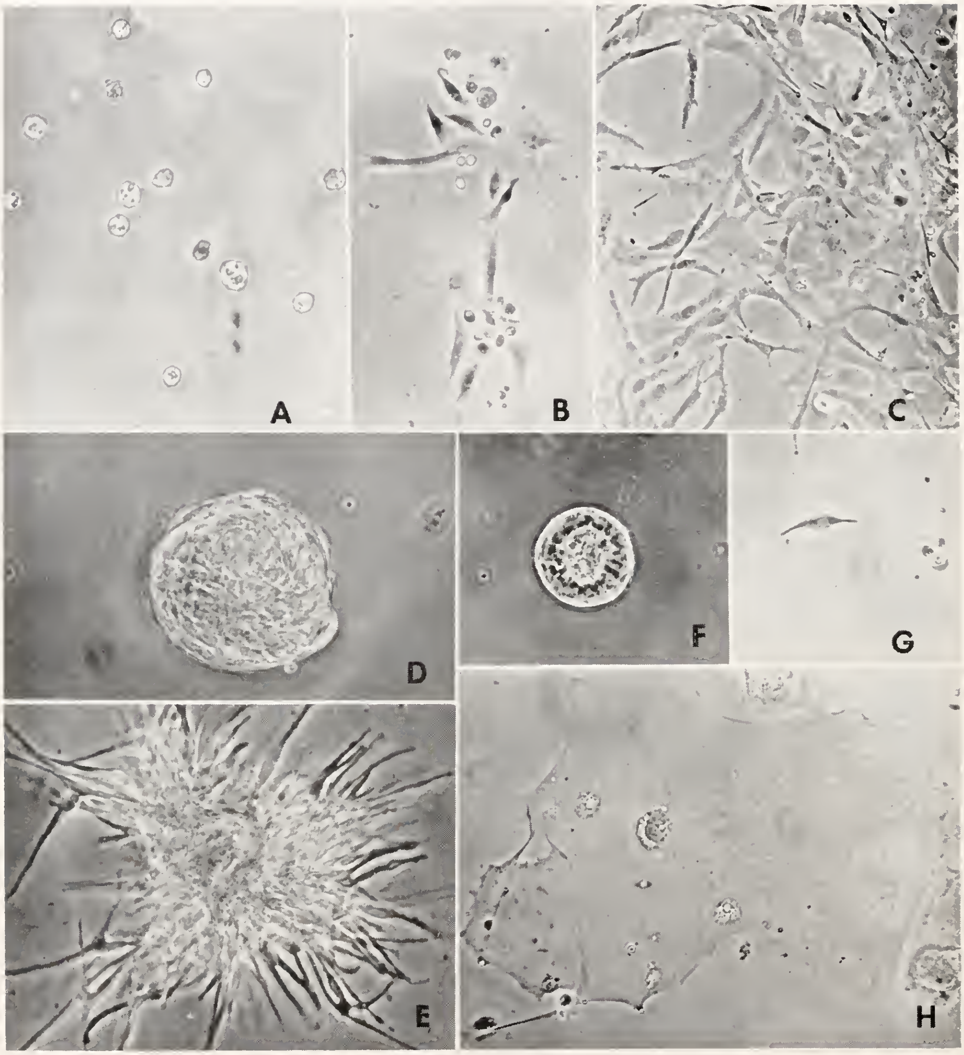

FIGURE 4, A.–H.

FIGURE 4 (A.–C.). Blood cells of previously injured Cynthia pupae cultured in Grace's medium, A. is after 15 minutes (490 ×), B. after 1 hour (290 ×) and C. after 7 days (290 ×). (D.) A developing cyst cultured for 2 days in Grace's medium which had been preconditioned for 24 hours by blood cells, the latter remaining in the culture. The developing sperm forms whorls within the non-elongating cyst (490 ×). (E.) A developing cyst as in Figure 4 D. except that in this case the cyst has spontaneously ruptured to reveal the differentiating spermatozoa (490 ×). (F.–H.) These three figures are reproduced at the same magnification (290 ×). F. is a normal germinal cyst from a diapauisng Cynthia testis. When subjected to osmotic shock, it dissociated into spermatocytes and follicle cells which were cultured for 10 days in Grace's solution containing a Cynthia plasma fraction precipitated by 3 M ammonium sulfate. The isolate spermatocytes (Fig. 4G.) survive but do not develop. By contrast, the isolated follicle cells proliferate and form giant cells illustrated in Figure 4H. For further details see text.

Grace's medium lacking plasma or hemocytes. Nevertheless, the development was abnormal in that the maturing cysts failed to undergo the elongation routinely encountered in cultures prepared in blood or plasma. They remained as spherical objects with the tails of the spermatids and spermatozoa forming whorls within the enveloping follicle cells (Fig. 4D and E). We suspect that this faulty development can be attributed to a failure of the medium to sustain the normal growth response of the follicle cells.

6. *MF in extracts of pupal fat body*

To determine whether other tissues contained MF, a series of Cynthia pupae 1 to 3 months after pupation was sacrificed and dissected in cold Grace's medium containing crystals of PTU. Masses of fat body were removed and thoroughly washed in the cold medium to remove nearly all hemolymph and hemocytes. The tissue was gently blotted, weighed, and approximately 1.5 g placed in 0.5 ml of cold Grace's medium in an all-glass homogenizer. After homogenization and centrifugation the clear supernatant was collected from between the precipitate and a superficial lipid layer. The precipitate was reextracted several additional times in small volumes of medium until a total of 2.5 ml of clear supernatant had been collected. The latter was sterilized by pressure-filtration through a Millipore filter (0.45 μ pore size) and placed in a 30 ml plastic culture chamber to which were added the germinal cysts obtained from two diapausing Cynthia testes. In a total of eight cultures of this type the cysts survived for at least six days but showed no development.

The experiment was repeated on a second group of Cynthia 1 to 3 months after pupation. In this case each pupa received a large integumentary injury to provoke the appearance of MF in the blood. During the first three days after injury the fat body of these individuals continued to show no detectable MF. However, by the sixth day after injury, considerable MF activity could be extracted from the fat body.

An additional experiment was performed on Cynthia pupae stored for 6 to 9 months after pupation. It will be recalled that the blood of these individuals contains high titers of MF (Fig. 2). So did their fat body when the latter was extracted and assayed in 5 of 6 cultures (20 to 70% development). By contrast, the intersegmental muscles and wing epidermis showed no extractable MF.

TABLE II

In vivo activation of the blood plasma of three species of diapausing pupae

Species of pupae donating plasma	Duration of diapause (weeks at 25° C)	No. pupae	Initial titer of MF	Titer 24 hours* after injury
S. cynthia	8	5	0 (10)**	43 ± 5 (16)**
H. cecropia	8	5	7 ± 7 (13)	55 ± 16 (7)
S. cynthia	15	6	27 ± 9 (27)	76 ± 12 (25)
A. mylitta	16	5	24 ± 6 (9)	78 ± 9 (9)

* All assays were on cysts of diapausing *S. cynthia* pupae.

** Number of cultures are recorded in parentheses.

From these preliminary studies it appears that, once MF is released into the blood, a certain proportion can be taken up by the fat body and sequestered.

7. *MF-like activity in mammalian sera*

In the case of insects as well as vertebrates the successful *in vitro* culture of cells and tissues in "synthetic" media has routinely required the addition of one or more macromolecular fractions (Wyatt, 1956; Eagle, 1955). For example, Grace's medium has generally been supplemented by 3 to 5% heat-treated (60° C for 5 minutes) hemolymph derived from diapausing silkworm pupae (Grace, 1962; Grace and Bryostowski, 1966)—a blood fraction which most likely contains substantial MF. Subsequently, it was found that hemolymph could often be replaced by other proteinaceous materials such as heat-treated calf serum (for detailed review see Brooks and Kurtti, 1971).

We were therefore encouraged to examine three commercially available mammalian sera for the presence of MF activity. Calf serum, fetal calf serum, and newborn calf serum (see section on METHODS) were heated at 56° C for 30 minutes to eliminate toxic components. The supernatants were then added in various proportions to Grace's medium and assayed for MF activity in plastic flasks containing germinal cysts derived from diapausing Cynthia pupae.

The results summarized in Table III reveal the surprising fact that all three mammalian sera stimulated the initiation of spermatogenesis when present in critical concentrations distinctive of each material. Meiosis took place accompanied by the beginning of elongation. However, development did not proceed beyond stage III and all cysts died and disintegrated after 7 or 8 days of culture. These findings suggest that the vertebrate sera contain one or more MF-like materials which cannot fully substitute for the authentic MF of silkworm blood.

8. *Precipitation of MF at low ionic strengths*

We have confirmed the unpublished findings of Dr. Melvin M. Ketchel (Tufts University Medical School) that MF can be precipitated from active plasma by the addition of critical amounts of distilled water. In the experiment in question 0.5

TABLE III

Flask cultures of cysts in Grace's medium supplemented with mammalian sera or Cynthia plasma

Sera	Number of cultures	% of cysts developing* as a function of % serum or plasma				
		20	40	60	80	100
Calf serum	20	6 ± 1	34 ± 9	21 ± 4	0	0
Fetal calf serum	15	10 ± 3	40 ± 8	20 ± 4	0	0
Newborn calf serum	50	16 ± 2	15 ± 5	61 ± 8	0	0
Active Cynthia plasma	30	0	30 ± 3	65 ± 7	80 ± 8	80 ± 7

* Each datum records per cent of cysts developing to Stages II and III; in the mammalian sera development never proceeded beyond Stage III.

TABLE IV

Fractionation of active blood plasma

Ratio hemolymph/H_2O	Number of cultures	% of cysts developing	
		Supernatant*	Precipitate**
1:0	15	61 ± 5	—
1:10	20	40 ± 6	0
1:20	20	32 ± 8	0
1:40	20	12 ± 3	20 ± 5
1:50	20	0	55 ± 6

* Lyophilized and redissolved in Grace's medium.

** Redissolved in Grace's medium.

ml of active Cynthia plasma was placed in each of four tubes. Double distilled water was added in volumes of 10, 20, 40, and 50, respectively. The tubes were incubated at 25° C for 30 minutes and then centrifuged at 17,600 *g* for 25 minutes. The supernatants were decanted, lyophilized, and redissolved in 0.5 ml of Grace's medium. The precipitates were also dissolved in 0.5 ml of Grace's medium. The osmotic pressure of each solution was determined and small amounts of additional Grace's medium (osmolarity 335 milliosmols) was added to lower the pressure to approximately 395 milliosmols. Each solution was sterilized by pressure-filtration through a Millipore filter and used to prepare 15 to 20 hanging-drop cultures containing germinal cysts of Cynthia pupae.

As summarized in Table IV, MF remained soluble after the addition of up to 20 parts water, but was partially precipitated in 40 parts water and fully precipitated in 50 parts water. The active precipitate permitted full development of the cysts including their elongation. When it was examined by disc electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (pH 7.2, 7.5% polyacrylamide, 0.1% SDS), one faint band and four dense bands were evident.

9. *Ammonium sulfate fractionation*

As a second approach to the purification of MF, 5 ml of active Cynthia plasma were dialyzed at 2° C against 1 liter of 2 M ammonium sulfate whose pH was adjusted to 6.8 at 2° C by the addition of sodium hydroxide. After 12 hours the non-dialyzable fraction was collected and centrifuged (20 minutes at 17,600 *g*), the precipitate being collected and temporarily frozen at −25° C. Further dialysis of the supernatant was carried out step-wise against 2.5, 3.0, 3.5, and 4.0 M ammonium sulfate (pH 6.8 at 2° C).

To eliminate residual ammonium sulfate each of the five precipitates was dissolved in 1 ml of 0.01 M phosphate buffer, pH 6.8, and dialyzed at 2° C for 24 hours against three changes of the same buffer. Each non-dialyzable fraction was lyophilized and made up to 2.5 ml with Grace's medium. The pH was adjusted to 6.8 at room temperature and the milliosmolarity to 395. Each solution was pressure-filtered through a sterile Millipore filter and then placed in a plastic culture flask to which the germinal cysts from two Cynthia testes were added.

MF activity was recovered only in the fraction precipitated in 3.0 M ammonium sulfate. In the culture containing this fraction, 30 to 40% of the cysts formed spermatids and spermatozoa. But here again (as in the case of cysts cultured in Grace's medium containing hemocytes) the cysts remained spherical and failed to elongate, the sperm flagella forming whorls within the cavity formed by the surrounding follicle cells.

In several experiments the cysts were first subjected to osmotic shock by exposing them to Grace's medium for about 0.5 hour. (As mentioned in Section 8, this medium is hypotonic in the absence of added serum or plasma fractions.) When the dissociated spermatocytes and follicle cells were cultured in Grace's medium supplemented by the MF-containing ammonium sulfate cut, dissociated spermatocytes survived for up to twenty days but failed to develop. Meanwhile, many of the dissociated follicle cells adhered to the plastic and remained viable for about a week; during this period they showed little growth and no multiplication. It is of particular interest that isolated spermatocytes did not respond to MF.

In cultures containing the four other plasma fractions lacking MF activity, the cysts remained intact and apparently healthy for several days; they then progressively dissociated into spermatocytes and follicle cells. Here again, the free germinal cells remained apparently healthy for up to twenty days but showed no development.

The behavior of the free follicle cells differed among the several cultures. In the presence of the plasma fractions precipitated by 2.0 or 2.5 M ammonium sulfate, a few of the cells adhered to the plastic; they showed slight growth but no multiplication, and usually died after 7 to 10 days. By contrast, in the presence of the plasma fractions precipitated by 3.5 or 4.0 M ammonium sulfate, the vast majority of the free follicle cells spread out on the plastic, increased in number by mitotic divisions, and then underwent enormous growth and polyploidization to form giant, flattened cells which remained healthy for as long as two months (Fig. 4F–H). These particular fractions evidently contained one or more components with the ability to promote the growth and development of isolated follicle cells but not of the germinal cysts as a whole.

Discussion

The normal milieu of the germinal cysts is the fluid which fills the testicular cavities (Fig. 1A). Interposed between this fluid and the surrounding hemolymph are the numerous cellular and membranous components which comprise the walls of the testes. All these barriers are automatically eliminated when the testes are torn open and the "naked" cysts are subjected to *in vitro* culture. Under this circumstance, the metamorphosis of the germinal cysts into bundles of spermatozoa was found to depend, not on ecdysone, but on an undialyzable, heat-sensitive, non–species-specific macromolecular factor which we have called MF.

As indicated in Figure 2, the titer of MF in the plasma can be described by a U-shaped curve when diapausing male or female Cynthia pupae are stored at 25° C for up to six months. No MF was detectable in assays performed on plasma collected from uninjured pupae during a brief period 8 to 10 weeks after pupation. These systematic alterations in titer are presumably attributable to differential changes in MF synthesis, compartmentation, and inactivation.

In Sections 4 and 5 of the RESULTS we considered the circumstantial evidence that one or more types of hemocytes may be the source of MF. Assays performed on whole blood routinely showed higher MF titers than the corresponding plasma. Moreover, when the hemocytes were collected from injured pupae and cultured in Grace's medium, they not only survived and multiplied, but also contributed to the medium an MF-like activity which stimulated the maturation of spermatocytes.

In cultures of this sort the germinal cysts failed to elongate despite the differentiation of spermatids and spermatozoa. This same abnormal development was encountered in cultures prepared in Grace's medium supplemented with an MF-containing plasma fraction precipitated by 3.0 M ammonium sulfate (see Section 9). When tested in this same manner, certain other ammonium sulfate fractions lacked MF activity but provoked a spectacular growth response of follicle cells isolated from the germinal cysts. Evidently, in addition to MF, the plasma contains at least one additional type of macromolecule prerequisite for the normal development of germinal cysts.

The successful culture of insect cells and tissues, as reported in the literature, has routinely required the presence of insect hemolymph or certain other macromolecular fractions derived from mammalian sera. It is therefore of considerable interest that MF-like activities were demonstrated in assays of heat-treated calf serum, fetal calf serum, and newborn calf serum. We are presently investigating the relation of these active materials to the "serum factors" prerequisite for the culture of mammalian cells (Eagle, 1955; Puck, 1961; Temin, 1967; Todaro, Matsuya, Bloom, Robbins and Green, 1967; Holley and Kiernan, 1968; Paul, Lipton and Klinger, 1971).

Whether MF constitutes a single molecular species can be determined only after its further purification and characterization. Further purification is also necessary to decide whether MF functions at catalytic or substrate concentrations.

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SUMMARY

1. "Naked" germinal cysts removed from the testes of diapausing Cynthia or Cecropia pupae and cultured *in vitro* undergo meiosis and spermatogenesis only when the culture medium contains a "macromolecular factor" (MF) which is present in insect blood. The factor in question is undialyzable, heat-sensitive, and interchangeable among the three genera of saturniid silkworms which were studied. Its partial purification was achieved by ammonium sulfate fractionation and by precipitation at low ionic strengths.

2. In appropriate experiments, ecdysone was found to have no obvious effect on cultures of naked cysts and was neither able to replace nor enhance the activity of MF in the *in vitro* assay.

3. MF is present in the blood plasma of both male and female pupae; its titer undergoes large and systematic changes when diapausing Cynthia pupae are stored at 25° C for up to six months.

4. In plasma collected from uninjured pupae, MF was routinely present except during a brief period 8 to 10 weeks after pupation. However, even in that case, substantial MF activity appeared in the blood during the first two days after an integumentary injury.

5. Circumstantial evidence is presented that MF is synthesized and secreted by one or more types of hemocytes when the latter are activated as, for example, by integumentary injury. Presumably because of their ability to secrete MF and thereby to condition the medium, the hemocytes were the only class of cell which could be cultured in Grace's medium without the addition of any macromolecules.

6. MF-like activities were demonstrated in assays of heat-treated calf serum, fetal calf serum, and newborn calf serum. It is not yet known whether these activities are related to the "serum factors" prerequisite for the successful culture of mammalian cells.

7. For these several reasons it is conjectured that MF or MF-like materials have heretofore been present in virtually all of the media which have sustained the successful culture of insect cells and tissues.

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IN VITRO DEVELOPMENT OF INSECT TISSUES. II. THE ROLE OF ECDYSONE IN THE SPERMATOGENESIS OF SILKWORMS[1]

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In the preceding paper of this series (Kambysellis and Williams, 1971) we confirmed the earlier observations of Schmidt and Williams (1953) that a biologically active "macromolecular factor" (MF) is present in the hemolymph of male and female silkworms. MF is recognizable in terms of its ability to provoke the meiosis and spermatogenesis of germinal cysts removed from diapausing testes and cultured *in vitro*.

By the use of this biological assay the blood of Cynthia pupae was found to contain substantial MF activity throughout all but a few weeks of pupal diapause. Yet, strange to say, the germinal cysts within the testes of diapausing pupae undergo no developmental response until such time as diapause is terminated in response to ecdysone injection or the secretion of ecdysone by the insect's own prothoracic glands.

This paradox is examined in detail in the studies reported here. Our experimental approach was to examine the effects of MF and ecdysone on *in vitro* cultures of intact testes. The results were strikingly different from those previously reported for the naked germinal cysts.

MATERIALS AND METHODS

All experiments were carried out on diapausing pupae of the Cynthia silkworm (*Samia cynthia*). The experimental procedures were precisely the same as described by Kambysellis and Williams (1971) except that the *in vitro* technique was modified for the culture of intact testes.

Each culture chamber consisted of a depression slide (spherical concavity 18 mm in diameter and 1.5 mm in depth), a Teflon spacer ring 2.5 mm in thickness (cut from 25.3 mm O.D., 19.0 mm I.D. tubing), and a No. 1 circular glass coverslip 22.0 mm in diameter. All components were sterilized overnight at 140° C.

To prepare the culture, 100 μl of an appropriate medium was pipetted into the concavity of the slide and an intact testis, cleaned-up and rinsed as previously described (Kambysellis and Williams, 1971), was added. The testis was handled by grasping an attached fragment of trachea with sterile forceps. The Teflon spacer ring was placed in position and capped with the cover slip. The assembled chamber was then sealed by brushing melted wax around its edges.

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The samples of α-ecdysone and phytoecdysones were obtained through the courtesy of Dr. John Siddall of Zoecon Corporation, Prof. K. Nakanishi of Columbia University, and Prof. T. Takemoto of Tohoku University. Mention is also made of an experiment using tritiated ecdysone; this material was synthetic α-ecdysone-23,24-^{3}H (40 c/mmole) which was most kindly supplied by Dr. Siddall.

RESULTS

1. *Development of germinal cysts* in vitro *and* in vivo

(a) *Effects of MF.* In a series of control experiments the titer of MF was assayed in hanging-drop cultures prepared from the blood plasma and naked germinal cysts of each of ten normal, diapausing, male Cynthia 2 to 3 months after pupation. As anticipated for pupae of this age-class (Kambysellis and Williams, 1971), the assays confirmed the presence of a low titer of MF as signaled by the development of only 5 ± 4% of the germinal cysts (Table I). The experiment was repeated on ten additional pupae which had been stored at 25° C for 5 to 6 months. As anticipated for pupae of this age, substantial MF activity was revealed by the assays (25 ± 5%).

Microscopic examination of the freshly prepared cultures revealed no trace of development of the cysts immediately after their removal from the testes. In these and scores of similar preparations from diapausing pupae, meiosis and spermatogenesis of naked cysts began only when they were cultured in direct contact with MF.

(b) *Effects of integumentary injury.* The experiment was repeated on twelve pupae that had been stored at 25° C for 2 to 3 months. In this case, each individual was transected with a razor blade just behind the metathorax to provide isolated

TABLE I

Effects of injury and of ecdysone injection on the development of the germinal cysts in vivo *and* in vitro

Treatment	Number of pupae	Hours after treatment	% of cysts developing		
			In the intact testes *in situ*	In cultures of the pupa's own cysts and plasma	In plasma cultures of cysts from untreated pupae
None (controls)	10	—	0	5 ± 4(53)†	—
	10*	—	0	25 ± 5(45)	—
Injured (abdomens isolated)	6	72	0	56 ± 5(18)	65 ± 9
	6	96	0	41 ± 7(18)	43 ± 6
Inject 10 μg α-ecdysone	5	1	0	—	3 ± 2
	5	24	[Stage II only]	—	25 ± 6
	5	48	10–20	83 ± 6(17)	33 ± 8
	5	72	40–50	86 ± 7(20)	45 ± 11

* These 10 pupae were 5–6 months after pupation; all the others were 2–3 months.
† Number of cultures are recorded in parentheses.

abdomens which had sustained massive integumentary injuries. Crystals of an equal part mixture of phenylthiourea and streptomycin sulfate were placed in the wound along with sufficient blood from the anterior fragment to displace all air. Each isolated abdomen was then sealed with melted wax to a plastic slip.

After 72 hours six of the preparations were sacrificed. The hemolymph and testes of each individual were collected and plasma cultures of the germinal cysts were prepared in the usual manner. In parallel assays an aliquot of each sample of plasma was tested for MF titer on germinal cysts obtained from normal, uninjured, diapausing pupae. These same manipulations were carried out on the second group of six abdomens which were sacrificed 96 hours after their isolation.

As indicated in Table I, all samples of plasma from the injured, isolated abdomens showed high MF activity irrespective of whether they were assayed on their own germinal cysts or on cysts obtained from uninjured diapausing pupae.

(c) *Effects of ecdysone.* Table I summarizes a further series of experiments carried out on twenty Cynthia 2 to 3 months after pupation. At zero hours each individual was injected with 10 μg of α-ecdysone. Groups of five individuals were sacrificed at specific periods after injection and treated as described above for the isolated abdomens.

The samples of plasma, when assayed on the germinal cysts of normal uninjected pupae, showed a substantial increase in MF titer within 24 hours and further increases during the succeeding two days. The cysts harvested from the testes of the injected pupae showed no development 1 hour after injection. But after 24 hours the testes contained many cysts which had completed meiosis (Stage II). By 48 to 72 hours after injection the testes contained steadily increasing numbers of cysts showing advanced spermatogenesis (Stages III and IV). As indicated in Table I, nearly all of these germinal cysts completed spermatogenesis when cultured for 7 days in the MF-containing plasma of the injected individuals.

These several experiments confirm a conclusion already documented by Kambysellis and Williams (1971)—namely, that naked cysts require MF for their development; as previously shown, the presence or absence of ecdysone is inconsequential in cultures of naked cysts. The present experiments go on to show that cysts within the intact testes of normal or injured pupae cannot respond to MF unless ecdysone is also present. These findings raised the possibility that ecdysone promotes the entry of MF into the cavity of the testes. This hypothesis was subjected to direct examination in the experiments which follow.

2. *In vitro culture of intact testes: effects of MF and ecdysone*

Individual testes of normal, diapausing Cynthia were cultured: (1) in medium containing MF but no ecdysone; (2) in medium containing ecdysone but no MF; and (3) in medium containing both MF and ecdysone. After 7 days the testes were torn open and examined microscopically for the development of the germinal cysts. As summarized in Table II, the cysts showed development only in those cultures containing both ecdysone and MF.

Table II summarizes a further series of experiments in which the agents were administered sequentially. For this purpose, individual testes were cultured for 1 hour in a medium containing ecdysone but no MF. They were then thoroughly rinsed and cultured for 7 days in medium containing MF but no ecdysone. In

Table II

*Spermatogenesis in intact testes of Cynthia pupae cultured with or without addition of MF and α-ecdysone**

Preincubation for 1 hour with:		Culture for 7 days with:	Number of testis cultures	Number of testes showing spermatogenesis	Developing cysts in responding testis (%)
—	Rinsed	MF	30	0	0
—		Ecdysone	21	0	0
—		Ecdysone + MF	30	30	40–80
Ecdysone		MF	12	12	20–50
MF		Ecdysone	12	0	0
Ecdysone + MF		No MF, no ecdysone	12	0	0

* α-Ecdysone, when added, was at a concentration of 1.6 μg per 100 μl medium.

parallel experiments the two agents were administered in reverse order. The results as summarized in Table II were clear-cut: development took place only when ecdysone came first.

In a final group of experiments the testes were cultured for one hour in medium containing both ecdysone and MF. They were then rinsed and cultured for 7 days in a medium lacking both ecdysone and MF. As indicated in Table II, no development took place.

3. *Ecdysone titer in relation to spermatogenesis*

Intact testes of diapausing Cynthia pupae were cultured in MF-containing plasma to which graded doses of α-ecdysone were added. After 7 days the testes were torn open and the development of the germinal cysts scored in the usual way.

As summarized in Table III, significant development invariably took place when the 100 μl of medium contained not less than 0.01 μg of α-ecdysone. The lower dose of 0.005 μg caused significant development in 2 of 4 testes, whereas the still lower dose of 0.001 μg was completely ineffective. The critical dose of 0.01 μg is equiv-

Table III

Spermatogenesis in intact testes of Cynthia pupae cultured seven days in plasma containing MF plus graded concentrations of α-ecdysone

Conc. of α-ecdysone (μg/100 μl)	Number of testis cultures	Number showing spermatogenesis	Developing cysts in responding testis (%)	
			Stages II and III	Stage IV
8	2	2	30–50	30–40
4	4	4	20–50	10–20
2	4	4	30–40	10–20
0.4	2	2	10–40	5–10
0.16	2	2	20–30	5–10
0.08	3	3	10–30	5–10
0.04	2	2	10–30	5–10
0.01	4	4	10–30	0–10
0.005	4	2	10–20	0–10
0.001	4	0	0	0

alent to a final concentration of one part α-ecdysone per ten million parts medium (2×10^{-7} M).

4. *Tests of other steroids and specific solvents in cultures of intact testes*

(a) *Effects of solvents.* Intact Cynthia testes were cultured in 100 μl of MF-containing plasma. Specific sterols were dissolved in one or more organic solvents, diluted to 10% by the addition of water, and 10 μl of the resulting solution administered to each culture.

In preliminary experiments of this type many organic solvents, despite their low concentration in the medium (*ca.* 1%), were found to have deleterious effects on the testes. Perhaps by injuring and thus altering the penetrability of the testis walls, the lower alcohols (see Table IV) allowed the entry of MF and the resulting onset of cyst development. These alcohols had no effect on cyst development if used in the absence of MF, whether with intact testes or with naked cysts. Solvent effects were overcome by the discovery that many of the steroids were soluble in

TABLE IV

Spermatogenesis in intact testes of Cynthia pupae cultured six days in plasma containing MF plus specific steroids[*] *or solvents*[†]

Additives	Number of testis cultures	Number showing spermatogenesis	Developing cysts responding testis (%)
None (control)	32	0	0
Methanol	2	Dead	
Ethanol	1	1	10–20
1-Propanol	2	2	5–10
2-Propanol	10	8	20–30[§]
Dioxane	10	2	10–20[§]
1,2-Propanediol	26	0	0
α-Ecdysone	32	32	30–80
β-Ecdysone	16	16	30–60
Cyasterone	8	8	20–40
Inokosterone	6	6	30–60
Ponasterone A	6	6	20–50
Ponasterone C	6	6	30–60
Rubrosterone	10	6	5–10[§]
"Triol"[‡]	10	0	0
Cholesterol	6	0	0
Estradiol-17β	4	0	0
Estriol	4	0	0
Progesterone	4	0	0
Aldosterone	4	0	0
Deoxycorticosterone	4	0	0

[*] The steroids were administered in concentrations of 0.5–2 μg per 100 μl medium.

[†] Solvents administered in final concentrations of *ca* 1%.

[‡] 5β-cholest-7-en-one,2β,3β,14α trihydroxy.

[§] Developed not beyond Stage II.

1,2-propanediol (propylene glycol) and that this solvent had no detectable influence on the cultures when present in a final concentration of 1%.

(b) *Effects of phytoecdysones.* As summarized in Table IV, β-ecdysone, cyasterone, inokosterone, ponasterone A, and ponasterone C were able to duplicate the effects of α-ecdysone on the intact testes. This result is of special interest since all these materials are known to be highly active in provoking adult development when injected into diapausing Cynthia pupae (Williams, 1968).

(c) *Effects of rubrosterone and "triol."* These two materials are known to be inactive when physiological doses were assayed by injection into diapausing Cynthia pupae (unpublished observations of C. M. W.). In the cultures of intact testes, the triol was inactive and rubrosterone showed only a trace of activity.

(d) *Effects of cholesterol and of mammalian hormones.* Since these materials (see Table IV) were insoluble in 10% 1,2-propanediol, a weighed amount of each was dissolved in absolute ethanol and an appropriate volume placed in a sterile centrifuge tube. The ethanol was evaporated in a stream of nitrogen and the material redissolved in MF-containing plasma. The latter was then used to prepare the cultures.

As indicated in Table IV all these materials were inactive when assayed on intact testes. They are also known to be inactive in ecdysone assays carried out on diapausing saturniid pupae (unpublished experiments of C. M. W.).

5. *Effects of simultaneously cultured brains and/or prothoracic glands*

The ecdysone requirement, as indicated in Table III, is fully satisfied when as little as 0.01 μg of α-ecdysone is added to the 100 μl of MF-containing medium in each culture. We sought to determine whether this critical level of ecdysone activity can be generated *in vitro* by the culture of appropriate endocrine organs. Attention focused on the brain and prothoracic glands since a long-standing, albeit unproven, principle of insect endocrinology is that ecdysone is synthesized and secreted by the prothoracic glands when the latter are activated by a hormone secreted by the brain (Williams, 1947, 1952; Possompès, 1953; Wigglesworth, 1952, 1957, 1964).

Previous studies of the *in vivo* activities of these organs in saturniid silkworms were helpful in the design of the present experiments. Thus, on the basis of this knowledge, the optimal sources of active prothoracic glands (*i.e.*, glands already activated by brain hormone) are mature larvae on the first day of cocoon construction (Williams, 1952) or, alternatively, diapausing pupae of *Antheraea polyphemus* or *A. pernyi* after storage at 5° C for longer than 10 months. In the case of potentially polyvoltine species such as *Samia cynthia, A. polyphemus*, and *A. pernyi*, brains and prothoracic glands in the inactive condition can be obtained from freshly pupated individuals reared under the short-day conditions which provoke the onset of pupal diapause (Williams and Adkisson, 1964). By contrast, the brains of these same species are active in freshly pupated individuals reared under the long-day conditions which avert the onset of diapause (Williams, 1969). In all species, a further routine source of active brains are diapausing pupae stored at 5–8° C for 3 months or longer (Williams, 1956). In the experiments reported here we have assumed that the activities of the brains and prothoracic glands *in vitro* were the same as the above-mentioned activities *in vivo*.

TABLE V

Cultures of intact Cynthia testes in MF-containing plasma: effects of simultaneously cultured Cynthia brains and/or prothoracic glands

Endocrine organs	Donors	Number of cultures	Number that developed	% Developing cysts in responding testes
[None]	[Controls]	15	0	0
Active brain	Non-diapausing pupae	9	0	0
Inactive prothoracic glands	Diapausing pupae	9	0	0
Active prothoracic glands	Larvae (1st day of spinning)	10	9	30–80
Inactive prothoracic glands plus inactive brain	Diapausing pupae	6	0	0
Inactive prothoracic glands plus active brain	Brains from non-diapausing pupae; prothoracic glands from diapausing pupae	6	4	10–40
Active prothoracic glands (homogenized)	Larvae (1st day of spinning)	6	0	0

Individual testes of diapausing Cynthia pupae were cultured in 100 μl of MF-rich plasma derived from Cynthia pupae that had been stored at 25° C for 5 to 6 months. After 3 to 6 days of incubation at 25° C, the testes were torn open and examined microscopically.

As summarized in Table V, no development took place in 15 control cultures. The same negative results were observed in cultures supplemented with either an active brain or a pair of inactive prothoracic glands. The combination of inactive brain plus inactive prothoracic glands was also ineffective. By contrast, nearly all

TABLE VI

Cultures of intact Cynthia testes in MF-containing plasma: effects if simultaneously cultured organs from two other saturated species

Endocrine organs	Donors	Number of cultures	Number that developed	% Developing cysts in responding testes
[None]	[Controls]	33	0	0
Thoracic or abdominal ganglia	Pernyi pupae (prolonged chilled)	12	0	0
Active brains	Pernyi or Polyphemus pupae (chilled 5 months)	25	0	0
Inactive prothoracic glands	Pernyi pupae (chilled 5 months)	3	0	0
Active prothoracic glands	Pernyi or Polyphemus pupae (prolongly chilled)	17	14	30–50
Inactive prothoracic glands plus active brain	Pernyi pupae (chilled 5 months)	2	2	50–80
Active prothoracic glands plus active brain	Polyphemus pupae (prolongly chilled)	7	7	50–80
Active prothoracic glands (sonicated)	Polyphemus pupae (prolonged chilled)	9	0	0

testes showed clear-cut spermatogenesis when cultured in the presence of active prothoracic glands or inactive prothoracic glands plus active brains.

Table VI summarizes additional experiments in which the testes of *Samia cynthia* were cultured in MF-rich Cynthia plasma along with organs obtained from pupae of two other saturniid species. Here again, spermatogenesis took place only when the cultures contained active prothoracic glands or inactive prothoracic glands plus active brains.

As recorded in the bottom line of Table VI, pairs of active prothoracic glands were ineffective when killed by sonication. In Table V a similar negative result is noted for active prothoracic glands that had been homogenized.

Discussion

Spermatogenesis in cultures of intact testes requires the presence, not only of MF, but also of ecdysone (Table II). By contrast, the germinal cysts, when removed from the testes and cultured in direct contact with MF-containing medium, do not require ecdysone (Kambysellis and Williams, 1971). These findings can fully account for the developmental reactions within the testes of normal or injured pupae (Table I). Thus, notwithstanding the presence of high titers of MF in the hemolymph, diapausing pupae show no trace of spermatogenesis until such time as ecdysone is injected or secreted by the prothoracic glands.

Particularly illuminating are the experiments summarized in Table II in which intact testes were subjected to the simultaneous or sequential administration of ecdysone and MF. Spermatogenesis took place only when the exposure to ecdysone either accompanied or preceded the exposure to MF. It is also of interest that the effects of ecdysone were persistent after 1 hour of treatment, whereas exposure to MF for at least 24 hours was prerequisite for the initiation of the developmental response (Kambysellis and Williams, 1971).

Evidently, the function of ecdysone is to alter the penetrability of the testis walls and thereby to facilitate the entry of MF and perhaps other blood-borne molecules into contact with the germinal cysts. In support of this "permissive" role of ecdysone we found that when testes were cultured in an ecdysone-free but MF-containing medium, the development of the germinal cysts could be provoked by puncturing the testes or by the addition of certain organic solvents which apparently damage the testis walls (Table IV).

Otherwise, the effects of ecdysone on intact testes was specific for α-ecdysone, β-ecdysone, and phytoecdysones known to be highly active *in vivo* (Williams, 1968). α-Ecdysone, as illustrated in Table III, was fully effective when administered in concentrations as low as 2×10^{-7} M.

Of further interest in this connection is a series of experiments (to be described in detail elsewhere) in which intact testes were cultured for 2 hours in the presence of very low doses (2×10^{-9} M) of tritiated α-ecdysone. The label was rapidly taken up by the testes and only a small fraction (15%) could be eluted into the medium when the testes were cultured for 12 hours in the absence of ecdysone. At the conclusion of the experiment 75% of the radioactivity was recovered in extracts of the testis walls, even though the walls account for a much lower fraction of the mass of the testis.

This selective binding, presumably to ecdysone "receptors" (Cherbas and

Cherbas, 1970), can obviously account for the long-lasting effects of even brief exposure to ecdysone (Table II). However, at the present time we are unable to state where this binding takes place in relation to the numerous cellular and membranous components of the testis walls. There is also insufficient information to decide whether the changes in penetrability involve the active or facilitated transport of MF, or the opening of channels for passive diffusion between cells.

In the absence of added ecdysone, the required level of ecdysone activity can be generated *in vitro* by the simultaneous culture of living, endocrinologically competent prothoracic glands (Tables V and VI). Since this activity failed to appear in the absence of activated prothoracic glands, these findings strongly support the view that the prothoracic glands synthesize and secrete one or more materials with ecdysone activity. The only reasonable alternative is that the prothoracic glands secrete an ecdysone precursor which can be converted into active hormone by the plasma or the reacting tissues—in this case the testis itself.

We have observed that active prothoracic glands were ineffective when sonicated or homogenized. This implies that the synthesis and secretion of ecdysone are synchronized and that little hormone is stored within the prothoracic glands. On the basis of the calibration of the *in vitro* system (Table III) the "stored" ecdysone in a pair of active prothoracic glands can be equated to less than 0.005 μg α-ecdysone.

As summarized in Tables V and VI, prothoracic glands which were known to be inactive *in vivo* were also inactive *in vitro*. However a most noteworthy finding was that inactive glands could be "turned on" by the addition to the culture of a living, endocrinologically competent brain.

Our interpretation of the experiments reported here as well as in the previous paper is summarized in Figure 1. The germinal cysts within the intact testis re-

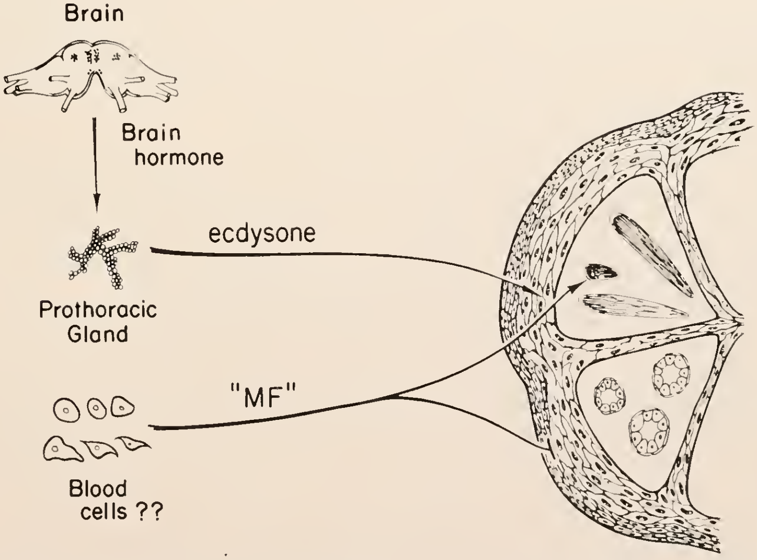

FIGURE 1. A diagrammatic representation of the control of spermatogenesis. See text for detailed description.

quire MF for their metamorphosis into bundles of spermatozoa. Though MF is ordinarily present in the surrounding hemolymph, it can gain access to the cavities of the testes only when the penetrability of their walls is altered in response to ecdysone.

MF is apparently synthesized and secreted into the blood plasma by one or more types of hemocytes when the latter are activated, for example, in response to injury of the pupal integument. Thus, as reported previously (Kambysellis and Williams, 1971), MF activity can be generated *in vitro* by culturing activated hemocytes in MF-free medium.

Finally, as diagrammed in Figure 1, the results of the present study show that ecdysone activity can be generated in an *in vitro* system containing living, endocrinologically active prothoracic glands. Moreover, endocrinologically inactive glands can be "turned on" *in vitro* by the further addition of living brains which are competent to secrete brain hormone.

The molecular mechanism of ecdysone action on the target cells of the testis walls remains, for the time being, a mystery. By analogy to what is known about the mode of action of the sterol hormones of vertebrates (Edelman and Fimognari, 1968; Gorski *et al.*, 1968; Fang *et al.*, 1969; Jensen *et al.*, 1969, Jensen *et al.*, 1971; O'Malley *et al.*, 1970; Steggles *et al.*, 1971), we would not be surprised to learn that the change in penetrability involves the *de novo* synthesis of one or more proteins.

Effects on permeability, including active transport and kindred phenomena, have often been cited as a mechanism for the implementation of hormone action (for review see Turner and Bagnara, 1971). However, in virtually all cases the altered permeabilities have pertained to water, ions, sugars, or other small molecules. Kroeger (1968) has presented evidence that ecdysone affects permeability relationships in the salivary glands of larval *Chironomus*. The effects in this case are thought to be on the active transport of sodium and potassium ions between nucleus and cytoplasm. The testicular system studied in the present investigation is therefore remarkable in that the ecdysone-induced change in penetrability is for a macromolecule which, in itself, possesses biological activity.

It is of interest that mammalian spermatozoa are known to differentiate in a fluid whose composition differs from that of the blood or lymph. The seminiferous tubules are surrounded by a "blood-testis barrier" which is virtually impermeable to serum albumin, inulin, and even to such small molecules as galactose and glutamic acid (Setchell *et al.*, 1969; Setchell, 1970; Dym and Fawcett, 1970).

It is tempting to speculate that penetrability barriers analogous to those of the testes may exist elsewhere in the insect body associated with cellular and extracellular layers such as the sheath of the nervous system or the basement membranes and mucopolysaccharide coatings of many cell types (for reviews see Ashhurst, 1968; Smith, 1968). Whether the penetrabilities of these other blood-tissue barriers may also be affected by ecdysone can be decided only on the basis of further study.

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SUMMARY

1. Germinal cysts, when removed from the testes and cultured in direct contact with the medium, undergo meiosis and spermatogenesis provided that a "macromolecular factor" (MF) is present.

2. By contrast, spermatogenesis within intact testes derived from either normal or injured pupae requires the presence, not only of MF, but also of ecdysone. The same is true for spermatogenesis *in vivo*.

3. The critical effects on the testes were exerted by concentrations of α-ecdysone as low as 2×10^{-7} M. The effects were shown to be specific for α-ecdysone, β-ecdysone, and phytoecdysones known to be highly active *in vivo*.

4. When MF and ecdysone were administered sequentially to cultures of intact testes, spermatogenesis took place only when the exposure to ecdysone preceded the exposure to MF.

5. The ecdysone requirement can be satisfied by the simultaneous culture of a pair of living, activated prothoracic glands. The glands were ineffective when homogenized or killed by sonication.

6. Prothoracic glands known to be inactive *in vivo* were also inactive *in vitro*. However, they could be "turned on" *in vitro* by the addition to the cultures of pupal brains known to be competent to secrete brain hormone.

7. These results strongly support the view that the prothoracic glands are activated by brain hormone to synthesize and secrete one or more materials with ecdysone activity.

8. Present indications are that ecdysone plays a permissive role in spermatogenesis and that its sole function is to alter the penetrability of the testis walls and thereby facilitate the entry of MF and perhaps other blood-borne molecules into contact with the germinal cysts.

9. Consideration is given to the possibility that the permissive role of ecdysone may not be an exclusive property of the testicular system.

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OSMOTIC CONSTITUENTS OF THE COELACANTH *LATIMERIA CHALUMNAE* SMITH

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The coelacanth *Latimeria chalumnae* occupies a unique position in the phylogenetic tree of the vertebrates as the only living representative of the crossopterygians, a group generally thought to lead from the ancestral bony fishes to the amphibians (Berg, 1958; Young, 1962; Romer, 1966). This, together with the fact that until 1939 the crossopterygians had been considered long extinct, dying out in the Cretaceous, accounts for the great interest shown in these "living fossils." However, in spite of an almost continual search since the end of the Second World War only two females and 30 males have been found to date, and there has been chemical analysis on only one specimen. On this fish, a large frozen male, Pickford and Grant (1967) analyzed the blood, Brown and Brown (1967) searched for the ornithine cycle enzymes and urea in the liver, and Cowgill, Hutchinson and Skinner (1968) looked at the mineral content of its hard and soft tissues. The offer to us by the Royal Scottish Museum of access to another specimen was therefore accepted with enthusiasm and it was thought worthwhile to confirm and perhaps extend the results on the first. As our fish was also frozen a small study was made to find the gross effect of prolonged freezing on the distribution of ions in a common fish (the freshwater perch) to help us interpret our data.

MATERIALS AND METHODS

Our specimen was a large male 32 kg weight and 1.37 m long which had been caught on March 1969 off the Grand Comore Island. It had been deep frozen and transported by air to Edinburgh. On the 5th of December (after nine months' freezing) two muscle cores and a liver core were taken from the still frozen body. On the 15th it was allowed to thaw and about 36 hours later a cast was made of the whole animal. After this the body cavity was opened and samples of body fluids and tissue taken. The fish appeared in good condition and there was no sign or smell of decay. Blood was taken from a mesentery blood vessel, the dorsal aorta, and a large dorsal blood vessel which was probably the vena cava. Tissue and body fluid samples were kept deep frozen (−20° C) until required for analysis.

The perch (*Perca fluviatilis*) used were killed by a sharp blow on the back of the head and were stored individually in sealed polythene bags at −20° C for six months. After being allowed to thaw, muscle samples were dissected from the epiaxial region and blood taken directly from the heart. The body fluid samples from both perch and *Latimeria* were centrifuged before use to remove all possible denatured protein "debris" and erythrocyte ghosts. Frozen tissues were allowed to thaw at room temperature and lightly blotted with filter paper; they were then weighed and the dry weight determined after heating in an oven at 105° C for 24 hours. From wet tissues 0.2 N HNO_3 and 1.0 N HNO_3 extracts were made for Na, K and Cl determination (Lutz, 1970). On the dry tissues 10% trichloroacetic acid extracts were used for Ca and Mg extraction (Lutz, 1970).

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The metal ions were measured on a Unicam atomic absorption spectrophotometer SP 90 with the individual standards approximating to the ionic composition (as far as the dominant ions are concerned) of plasma and tissues extract. For Ca and Mg 0.75% EDTA was added to both samples and salines (Cook, 1969).

Chloride was measured by the electrometric method of Cotlove (1964) using a Cotlove Automatic Chloride Titrator. Osmotic pressure was measured as sample freezing point using the Ramsay-Brown apparatus (Ramsay and Brown, 1955) and by the Krogh-Baldes vapor pressure method (Krogh, 1939, page 211).

The nitrogenous compounds of muscle were estimated on tungstic acid extracts, 5 ml 10% sodium tungstate and 5 ml $\frac{2}{3}$ N sulfuric acid for each 2 g fresh muscle. For serum and bile one volume of each of the tungstic acid reagents was added to one volume of fluid diluted with 1–3 volumes distilled water. Microdiffusion methods of Conway (1962) were used for ammonium ions of body fluids and muscle extracts, for urea (urease method) and for the final determination of total non-protein nitrogen after micro-Kjeldahl digestion of samples of the filtrates. The α-amino N of free amino acids was determined according to Frame, Russell and Wilhelmi (1943) and Russell (1944), being corrected for the color given by the ammonium ions (90% of that of α-amino N of glycine). Trimethylamine oxide (TMAO) was estimated on tungstic acid filtrates after Kermack, Lees and Wood (1955), formaldehyde being used to hold back ammonia during the micro-diffusion of the trimethylamine. Tungstic acid filtrates were also used in the estimation of creatinine (Owen, Iggo, Scandrett and Stewart, 1954), and creatine, the latter being converted to creatinine at pH 2.2 on a boiling water bath for 2 hours.

RESULTS

(a) *Effect of prolonged freezing on perch*

The results obtained from analysis of muscle and blood samples from three individuals frozen for six months are compared to the mean values for 30 normal fish (Table I). It can be seen that considerable, but uniform, changes have occurred. In blood Na has fallen to about half the *in vivo* value and shows a large rise in the frozen muscle. The fall is even greater for plasma Cl and the muscle Cl has doubled. Potassium has increased 20 fold in the post-mortem blood. Muscle potassium on the other hand has decreased steeply and the fact that it is now close to the plasma value suggests that in six months freezing K has almost equilibrated throughout the two compartments. Calcium behaves like Na with a fall in plasma and rise in muscle. A three-fold increase is seen in plasma Mg with, however, a correspondingly slight fall in muscle, leaving the ion concentration gradient between the two compartments by far the highest for this ion. It would appear that Mg is the least mobile of all the ions considered under these circumstances. No clear trend is apparent from this data for changes in tissue hydration.

To summarize, there is a general move to the equalization of concentrations

TABLE I

Effect of prolonged freezing on the inorganic constituents of perch blood and muscle (mM/l body fluid and mM/kg tissue water)

		Na	K	Ca	Mg	Cl	% water
Plasma	Normal*	154.2	3.6	4.38	1.44	120	—
	1	68.9	60.1	2.90	4.1	56.1	—
Serum	2	71.0	63.5		4.8	27.3	—
	3	78.8	63.8	1.50	4.5	39.2	—
	Normal*	19.9	143.0	2.6	15.1	11.6	80.2
	1	24.2	62.0	3.6	12.2	20.7	81.4
Muscle	2	30.6	53.8	2.4	11.5	16.4	80.1
	3	41.0	69.9	4.9	15.6	27.5	74.9

1, 2 and 3 refer to samples from frozen perch.
* n = 30.

during prolonged freezing, with, in six months, plasma Na and Cl falling to around half *in vivo* values and invading muscle tissue, K equilibrating throughout the body, and Ca and Mg showing similar shifts down concentration gradients. As thawing took no more than six hours at room temperature the bulk of the ion movement probably occurred in the frozen state.

(b) *Measurements from frozen* Latimeria

The results of analysis of various *Latimeria* body fluids for total osmotic pressure and osmotic constituents are seen in Table II. The actual ionic values are quite different from those reported for any living vertebrate and have obviously been influenced to an important extent by freezing. A wide variation is seen for most parameters and the two most important factors causing this would be unequal ion migration during post-mortem processes and the possible dilution of some body fluids by ice crystals formed during the prolonged freezing of the tissues. The latter phenomenon would primarily affect the osmotic pressure and total ion concentration in the fluids while increasing slightly the osmotic concentration in parts of muscle and other tissues. In the blood and coelomic fluid the total ion content varies directly with the osmotic pressure and, since in fishes the initial *in vivo* values for these fluids are similar (Lutz, 1970), the large variance found for frozen *Latimeria* most likely reflects dilution. If this is so then the higher values are probably the most reliable *i.e.*, those values around 1000 milliosmoles. In perch the bile has an osmotic pressure similar to that of plasma (Lutz, 1970) and if this is generally true of fishes then the results found here would further support the suggestion that the lower osmotic and total ion values in Table II result from ice crystal formation. It seems probable then that the vena cava samples are further complicated by dilution factors. Comparing with the values from frozen perch it is seen that in our *Latimeria* blood there is a higher Na and Cl, similar K and similar Ca.

The nitrogenous constituents of *Latimeria* body fluids show that the vena cava has also the lowest value of urea. Urea is clearly an important constituent with

Table II

Osmotic constituents of Latimeria *body fluids*

	Dorsal aorta serum	Vena cava serum	Mesentery vessel serum	Coelomic fluid	Bile
Na	88.6	89.5	135.4	101.4	73.8
K	62.5	35.6	60.2	66.5	58.2
Ca	1.74	1.00	2.30	2.9	1.75
Mg	3.81	1.56	9.44	11.7	95.8
Cl	86.53	88.44	125.4	105.4	77.5
Urea	337	242			289
Trimethylamine oxide		109.4			107.0
Total NPN mg-atoms/l		786			1224
Total inorganic ions	243.2	216.1	332.7	287.9	215.3
Osmotic pressure milliosmoles	968	766	1138		1135

values ranging from 240–340 mM/l. Trimethylamine oxide is also present in large amounts with concentrations just less than half those of urea and accounting from some 10–15% of the total non-protein nitrogen found. Free trimethylamine was not detected in plasma samples indicating that decomposition had not occurred to any significant extent.

Table III shows the osmotic composition of muscle and liver samples. As with blood the ion results are quite uncharacteristic of vertebrate tissue indicating considerable post-mortem changes. The consistency of the results as shown by the standard errors is, however, much better.

The liver differs from muscle principally in the very low water content (due to an extraordinary high fat content) and in the much higher values for Na and Cl.

Table III

Osmotic constituents of Latimeria *muscle and liver (mM/kg tissue water)*

	Muscle		Liver	
	Mean	Number	Mean	Number
Na	30.63 ± 2.294	8	98.53 ± 4.673	3
K	73.50 ± 8.365	6	50.10 ± 11.67	3
Ca	1.77 ± 0.253	6	1.94 ± 0.358	3
Mg	14.36 ± 1.143	7	9.53 ± 0.632	3
Cl	35.32 ± 4.73	5	115.5 ± 2.201	3
Urea	421.5 [378–465]	2		
Trimethylamine oxide	290	1		
Creatine	31.9 [25.6–38.3]	2		
Creatinine	1.85 [1.5–2.2]	2		
Amino acids	60.35 [50.7–70.0]	2		
Total NPN mg-atoms/kg water	1423 [1192–1654]	2		
% water	73.66 ± 1.51	5	48.94 ± 2.21	3

[ ] = range.
± = standard error.

High concentrations of these two ions are characteristic of the livers of a variety of vertebrates including mackerel (Becker, Bird, Kelly, Schilling, Soloman and Yound, 1958), perch (Lutz, 1970) and the rat (Widdowson and Dickerson, 1964), and this has been interpreted as indicating a common pattern of high intracellular values of Na and Cl for this tissue (Lutz, 1971). It seems likely that this generalization can be extended to the coelacanths.

The nitrogenous constituents further illustrate the importance of urea and trimethylamine oxide, both being found in muscle in significantly higher concentrations than in the body fluids. As in all vertebrates creatine and creatinine were found, the former presumably present partly as creatine phosphate *in vivo*. No free trimethylamine was detected.

DISCUSSION

A comparison of these results with those from the only other *Latimeria* examined so far is of interest (Table IV). Considering their history, data from both specimens agree quite well in values for K_p (plasma K) and K_m (muscle K), urea_p, mOsm_p, Na_m and Mg_m; and this together with the very high Mg_p found by Pickford and Grant (1967) suggests that considerable post-mortem ion shifts had also occurred in the first specimen. The results disagree strikingly in Na_p and Cl_p, with Pickford and Grant's specimen having almost double the values that we find. Although trimethylamine oxide was not looked for in the first *Latimeria*, the results of Pickford and Grant (1967) appear to exclude its presence in significant amounts, finding as they report, that urea made up 88% of the total blood NPN. The method of analysis used in this study is however quite specific and we have

TABLE IV

A comparison of the data of the blood and muscle of two frozen specimens of Latimeria (*mM/l blood, mM/l tissue water*)

Constituent	Blood serum		Muscle	
	1*	2[x]	1	2[+]
Na	104.5	181	30.6	29.3
K	52.8	51.3	73.5	106.6
Ca	1.68	3.5	1.8	2.7
Mg	4.94	14.4	14.4	10.0
Cl	100.1	199.0	35.3	0.4
Urea	290	355	422	
Trimethylamine oxide	109.4		290	
Total NPN	786	959	1423	
Milliosmoles	957	1181		

* Arithmetical means from Table II.

[x] Pickford and Grant (1967).

[+] Cowgill, Hutchinson and Skinner (1968).

The TMAO and total NPN values of 1* serum are single values for vena cava blood which is apparently diluted. Calculations from the urea concentrations of vena cava and dorsal aorta sera suggest TMAO value of 152 mM and a NPN of 1095 mg-atoms for the dorsal aorta blood, which are probably closer to the *in vivo* values.

confidence in these results. The very low values reported by Cowgill *et al.* (1968) for Cl are extraordinary and must be regarded as spurious, since they would indicate that this animal had a negligible to zero extracellular space in its muscle tissue. Perhaps the method used of x-ray fluorescence is not particularly applicable to chloride analysis in these systems.

The range of osmotic pressure values found for our more reliable samples is 968–1138 milliosmoles, similar to that judged best by Pickford and Grant (1181 mOsm). It is probable that these cover the *in vivo* value for the animal and point to it being in approximate osmotic equilibrium with sea water. Whether it is slightly hyper-osmotic (like the sharks) or hypo-osmotic is not possible to say. Pickford and Grant give a value of 1090 milliosmoles (derived from salinity determinations of Dr. M. S. Gordon) for sea water in the region of Grand Comore Island.

The amounts of urea and trimethylamine oxide found in blood and muscle are considerable, and the muscle values as far as we can find, are by far the highest yet recorded for any animal. This may be of some significance as post-mortem changes are most likely to be accompanied by a decrease in both constituents.

As far as the inorganic ions are concerned similar post-mortem changes to those found in perch undoubtedly have occurred as they would account for the very high K_p and Mg_p found in *Latimeria* blood and for the fact that K_p approaches K_m.

The very low Na_p and Cl_p found by us would also agree with this suggestion, and if true would mean that the Na_m and Cl_m are *in vivo* substantially less than the values shown here.

It would appear that the coelacanth is similar to the elasmobranchs and teleosts in having internal salt concentrations much less than that of the surrounding sea water ($\frac{1}{3}$–$\frac{1}{2}$ S.W.). Like the elasmobranchs, and in contrast to the teleosts, they are also in approximate osmotic balance with their environment, the difference in salt concentration being made up in both cases by the nitrogenous compounds urea and TMAO. It seems that teleosts are ureogenic, having the full complements of ornithine-urea cycle enzymes (Huggins, Skutsch and Baldwin, 1969) although this had previously been denied (Brown and Cohen, 1960). Their ancestors were probably also ureogenic.

The first crossopterygians and dipnoans were probably ureogenic as are their present-day descendants including *Latimeria* (Brown and Brown, 1967) and *Protopterus* (Janssens and Cohen, 1966). While most crossopterygians remained in fresh water, one line leading to the amphibians and hence all higher vertebrates, a side-branch, the coelacanths, invaded sea water in the Triassic (Romer, 1966). It is suggested that this group became adapted to the marine environment by the same basic methods as the elasmobranches had evolved in the Silurian, *i.e.*, by developing a physiological tolerance to high urea and TMAO concentrations, and evolving mechanisms for the active retention of both components. Both groups are therefore ureosmotic.

We are indebted to Dr. S. M. Andrews and Dr. A. S. Clarke, The Royal Scottish Museum, Edinburgh, for the opportunity of taking samples of body fluids from the coelacanth and for initially taking cores of muscle and liver from the frozen specimen.

SUMMARY

1. Samples of blood, bile, muscle and liver from a frozen specimen of the coelacanth *Latimeria chalumnae* were analyzed for ions and nonprotein nitrogenous compounds.

2. Mean values (mM/l) for blood serum (hemolyzed) and bile (figures in brackets) were Na 104.5 (73.8) K 52.8 (58.2), Ca 1.68 (1.75), Mg 4.49 (4.0), Cl 100.1 (77.5), urea 290 (289), trimethylamine oxide 109.4 (107.0), total NPN 786 mg-atoms (1224), osmolality 957 milliosmoles (1135).

3. Mean values (mM/kg water) for muscle and liver (figures in brackets) include Na 30.6 (98.5), K 73.5 (50.1), Ca 1.8 (1.9), Mg 14.3 (9.5), Cl 35.3 (115.5), urea 422, trimethylamine oxide 290, total NPN 1423.

4. A study of the effect of prolonged freezing on the electrolyte distribution of the perch *Perca fluviatillis* was made and compared with the above results.

5. *Latimeria* differs from a marine teleost and resembles an elasmobranch in having large quantities of urea and trimethylamine oxide in both blood and muscle, and a high osmotic concentration near that of sea water.

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TEMPERATURE EFFECTS ON THE DEVELOPMENTAL RATE OF SQUID (*LOLIGO PEALEI*) EMBRYOS[1]

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As is usual for invertebrates, the rate of embryonic development of some cephalopods appears to be temperature dependent. Hamabe (1960) reported total developmental times of 36–43 days at 13–17° C and 46 days at 10–12° C for *Loligo bleekeri.* Developmental time of *Loligo opalescens* ranged at least from 22–24 days at 16° C (Fields, 1965) to 30–35 days at 13.6° C (McGowan, 1954). Choe (1966) reported that the developmental times of five species of squid and cuttlefish were highly dependent on water temperature. Costello, Davidson, Eggers, Fox and Henley (1957, pages 155–159) warned that their timetable of development for *Loligo pealei* might vary considerably with water temperature.

The squid, *L. pealei,* comes inshore near Woods Hole, Massachusetts in April and remains through November (Sumner, Osburn and Cole, 1913; Summers, 1968, 1969, 1971 and unpublished). Squid eggs are usually available in the Woods Hole area from May to October (Verrill, 1882; Bumpus, 1898; Drew, 1911; Summers, 1968, 1969, 1971 and unpublished) during which time sea water temperatures range from approximately 10 to 23° C, with a maximum in mid-August. Salinity range is approximately 30–32‰ during this period. The common occurrence of squid eggs and the large, natural temperature range facilitated a study of the relationship between sea water temperature and the developmental rate of *L. pealei* at Woods Hole.

Materials and Methods

Squid, *L. pealei,* eggs were collected within 20 km of Woods Hole by otter trawl on the following dates in 1970: May 11, May 14, May 20, July 14, and September 24. Some eggs, deposited in laboratory tanks by freshly caught squid, were collected on: May 27, June 4, June 8, July 30, and August 13. Dr. John M. Arnold donated the eggs of July 30 and August 13. A total of 18 sets of egg strings were obtained by otter trawl and 19 sets were obtained from laboratory tanks. The source of squid eggs was not judged important because only healthy embryos were used in our experiments.

Squid egg strings contain 150 to 200 embryos each (Williams, 1909). Individual egg strings were examined, compared to Arnold's (1965) normal embryonic stages and grouped by developmental stage. Each set (4 to 6 egg strings from the same source and matched for developmental stage) was provided flowing sea water in a new 20 cm stacking dish lined with an open-ended cylinder of 1.4 mm nylon mesh. The mesh extended 5 to 10 cm above the rim and retained egg strings and freshly hatched squid.

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Ambient temperature sea water, from the laboratory sea water system, was supplied to stacking dishes through glass and rubber tubing. Chilled sea water, from the laboratory chilled sea water system, filled a seasoned fiberglass reservoir. Glass and rubber tubing siphons carried water from the reservoir to stacking dishes. Sea water flow was sufficient to circulate egg strings in each dish. In our experiments, nearly all eggs provided with flowing sea water hatched. Development terminated in a few cases when the sea water flow stopped and the water became stagnant. These sets were discarded and the data were not included in our results.

One randomly chosen egg string was removed from each dish and observed under a dissecting microscope on an average of once every 36 hours. At each observation at least 20 embryos were staged using Arnold's (1965) scale of stages; time, sea water temperature, and dominant developmental stage were recorded. Stage 30 (hatching) was recorded at the first occurrence of newly hatched squid. Seventeen sets of egg strings were observed in ambient temperature sea water. Twenty additional sets were sub-divided into ten matched pairs: one set of each pair in ambient sea water and one in chilled sea water. We made a total of 377 observations which represent approximately 7540 determinations of embryonic stages.

RESULTS

Plots of developmental stage *vs.* time before hatch were prepared for each set of egg strings; these were compared and grouped by similarity of developmental time course. Three distinct groups resulted, each with a specific temperature range as shown in Figure 1. Group I included all ten sets of egg strings from chilled sea water and nine sets from ambient sea water. Embryos in chilled sea water at 13.0 to 16.9° C exhibited a time course of development indistinguishable from that of embryos in ambient sea water at 12.0 to 18.0° C. Mean developmental time for Group I embryos was 642 hr. The ten sets of egg strings in Group II were kept in ambient sea water at 15.5 to 21.3° C; mean developmental time was 445 hr. Group III included eight sets of egg strings in ambient sea water at 21.5 to 23.0° C; mean developmental time was 257 hr. In all groups survivorship to hatching approached 100%.

Embryonic development appeared to consist of four phases. The first three phases each required a specific proportion of the total developmental time independent of temperature, but the fourth phase (hatching) required a specific time interval independent of temperature. Development to stage 12 was non-linear and required 13.2% (SD 3.4%) of the total developmental time. Embryos developed from stage 12 to stage 26 at a linear rate which occupied 51.3% (SD 5.5%) of the total time. Linear development from stage 26 to stage 29 required 22.0% (SD 3.2%) of the total time. Development from stage 29 to stage 30 (hatching) apparently required 52.0 hr (SD 4.0 hr) independent of temperature.

Figure 2 shows the total developmental time (deposition to hatching) plotted against sea water temperature for all data groups. Smooth curves were fit by inspection. The following temperature indices are included: maximum temperature, mid-range temperature, weighted mean temperature, and deposition temperature. The latter corresponded closely with the minimum temperature for each group because the sea water was warming seasonally during the experiments. Also included in Figure 2 are data from the literature on *L. pealei* by Arnold (1965), Bruce (1886) and Costello *et al.* (1957).

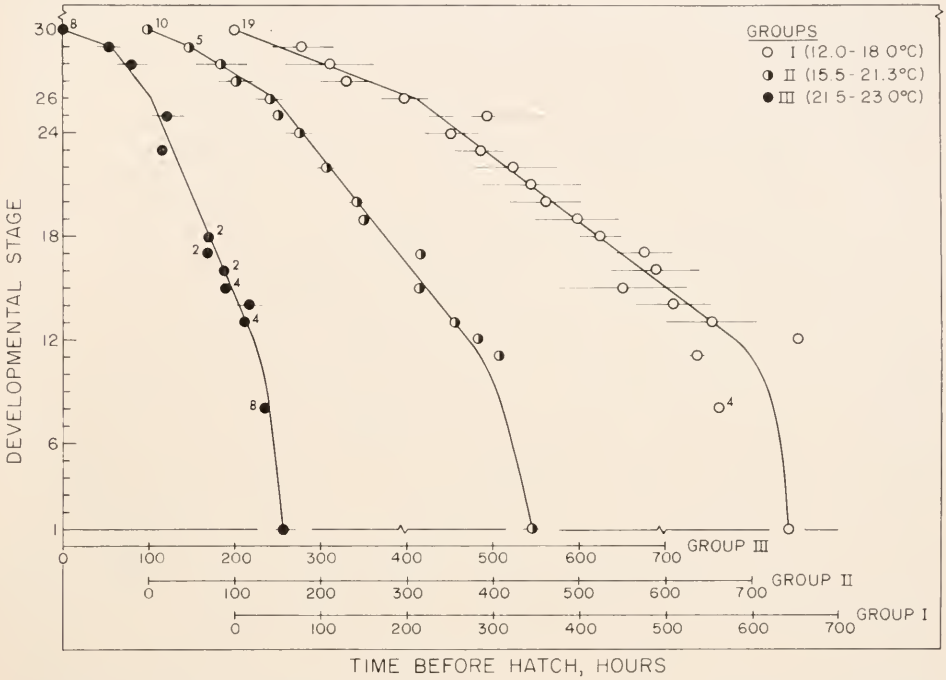

FIGURE 1. Mean and standard deviation of time before hatch at observed developmental stages (after Arnold, 1965) for each data group. The number of observations per point ranged from 1 to 33, with a mean of 7. Where more than one observation of a developmental stage occur at the same time before hatch, the number of such observations is shown beside the point. The horizontal scale has been displaced 100 hr between groups for clarity in presentation.

DISCUSSION

Our data show a direct relationship between sea water temperature and developmental rate to stage 29. The plot of deposition temperature *vs.* total developmental time (Fig. 2) is useful for predicting the approximate hatching time of squid embryos in seasonally warming sea water. If the deposition temperature is not known or if the sea water temperature is altered artificially (*e.g.*, when eggs are placed in chilled sea water), the approximate hatching time can be predicted by extrapolation from an observed time interval between established stages. At low temperatures, observed time of first hatch may differ from the predicted value by as much as 2 or 3 days due to heterochrony within individual egg strings.

The scatter of data summarized in Figure 2 does not permit extensive comparisons of developmental rate with published information on other animals, largely because of variation in ambient sea water temperatures. In our experiments, the total developmental time was approximately a linear function of mid-range temperature, and this relationship can be extrapolated to (an impossible) "zero" developmental time at about 27° C. Extrapolation of the seasonally varying deposition temperature suggests a minimum developmental time at nearly the same temperature. We cannot predict a temperature for "zero" developmental rate due to lack of data at temperatures below 12° C. Attempts to calculate the Bělehrádek

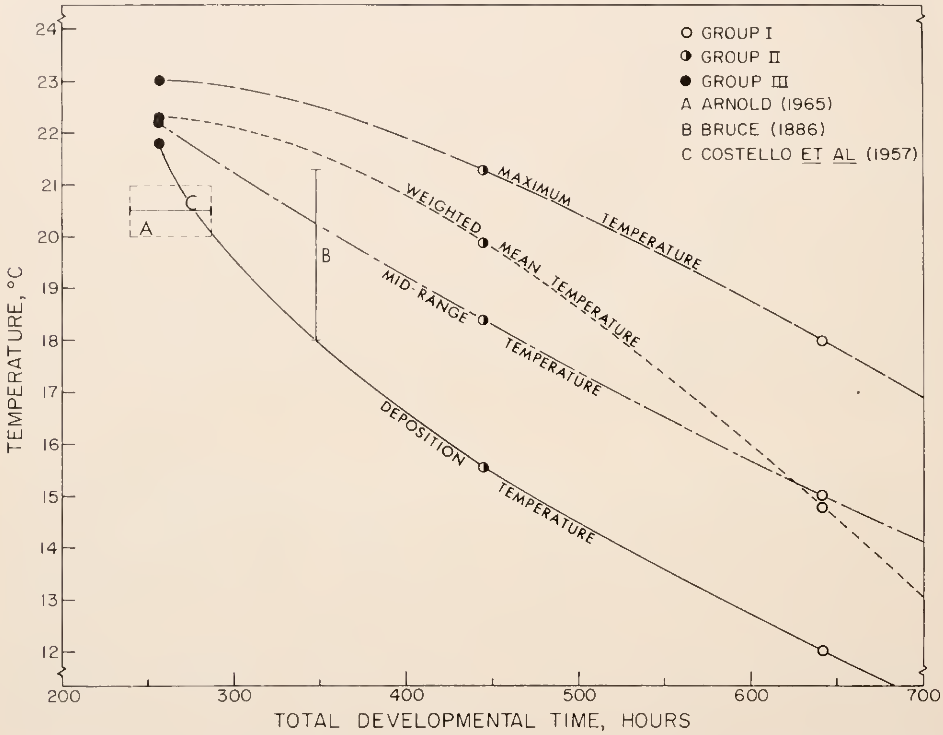

FIGURE 2. Total developmental time of *Loligo pealei* embryos as related to sea water temperature. Data from Arnold (1965), Bruce (1886) and Costello *et al.* (1957) are also included. Arnold (dashed box) reported a total developmental time of 240 to 288 hr for embryos at 20 to 21° C. Bruce (vertical line) reported that eggs deposited on July 3 hatched on July 18 (1886?), a total developmental time of approximately 348 hr. Costello *et al.* (horizontal line) reported a total developmental time of 240 to 288 hr. Bruce and Costello *et al.* provide no temperature data for their work at Woods Hole. Bruce's data are plotted between the temperatures (18.0° C, 21.3° C) observed on July 3 and July 18, 1970. Data from Costello *et al.* are plotted at the temperature (20.5° C) observed on July 15, 1970, because of the statement that most female squid have bred by mid-July.

"biological zero" temperature as used by McLaren (1966) were inconclusive. We do suggest that the natural occurrence of squid eggs would be limited to deposition temperatures at which adult squid are likely to be found, especially temperatures of at least 8° C (see Summers, 1969 and 1971). Thus, *L. pealei* eggs may fully develop over nearly a 20° span of sea water temperature, though we have verified this only over an 11° range.

The chronology of developmental stages at various sea water temperatures requires further examination. With the exception of early stages (corresponding to cellular events now classical in embryology) and hatching, Arnold's (1965) descriptions of normal embryonic stages were based on arbitrarily chosen, visible features not necessarily separated by consistent time intervals. As a result, stage rates of development should only be compared at the same stage between sets of embryos or over a number of consecutive stages for any one set of eggs. The latter is our justification for indicating linear stage rates and phases for portions of the data shown in Figure 1. As reported above, the developmental phases progressed in proportion to the total developmental time for all temperature groups with the exception of phase 4 (hatching). The inconsistency may be trivial because stage 30 was experimentally difficult and its first appearance probably represents a minimal hatching time.

Arnold's (1965) stage chronology clearly indicates a significant change in stage rate around stage 12 corresponding to the intersection of our phases 1 and 2. His data do not confirm our separation of phase 3 at stage 26. Broadly speaking, our phases relate to the following embryonic occurrences: (1) cellular events on the yolk surface, (2) differentiation, (3) growth at the expense of yolk and (4) hatching. The organic content of squid embryos has been reported by Russell-Hunter and Avolizi (1967). They demonstrated a relatively small increase in nitrogen and organic carbon, a greater relative increase in water content (wet weight minus dry weight) and a relatively large increase in salt content (inorganic ash) during the development of *L. pealei.* No significant uptake was reported during phase 1. The stage rate of salt uptake relative to water content dropped considerably between phases 2 and 3 possibly indicating the initiation of organ functioning and/or ionic regulation. This change is reflected in post-hatched values of 65% for organic components (carbon and nitrogen) and 21% for inorganic ash compared with stage 29 (Russell-Hunter, personal communication). These data suggest a functional distinction of phase 3.

Verrill (1882), Costello *et al.* (1957), Arnold (1965 and personal communication) and Russell-Hunter (personal communication) observed hatching of advanced (stage 29) *L. pealei* embryos caused by a mechanical stimulus. Fields (1965) considered low light intensity important for hatching of *L. opalescens.* Choe (1966) reported that a mechanical stimulus or a sudden change in either water temperature or salinity could elicit hatching in five cephalopod species. We observed hatching of advanced *L. pealei* embryos probably caused by an increase in water temperature or by a mechanical stimulus, but we did not specifically study any external hatching stimulus.

Squid in the vicinity of Woods Hole breed primarily from mid-May through June but some breeding continues into September (Verrill, 1882; Costello *et al.,* 1957; Summers, 1968, 1969, 1971 and unpublished). Due to sea water temperature differences, eggs deposited in May develop more slowly than eggs deposited in June. (In our experiments, eggs deposited on May 22 and June 3 hatched on June 18 and 21, respectively.) In an attempt to relate developmental temperature effects to the observed size distribution of young squid (Summers, 1967 and 1971), artificial size distributions were constructed by a Monte Carlo Method. Hatching size was assumed to be 1.8 mm dorsal mantle length which was the mean value from 88 measurements of newly hatched squid on two separate dates and corresponds with Arnold's (1965) scaled drawing of stage 30 squid. We assumed a normal distribution of egg deposition and a linear growth rate after hatching. Dates of mean egg deposition were selected at two-week intervals from May 15 to June 30. Sea water temperature data for Woods Hole (kindly provided by Mr. Charles L. Wheeler of the National Marine Fisheries Service, Biological Laboratory, Woods Hole) was used with Figure 2 to predict hatching dates. Constructed size distributions were skewed toward smaller animals; observed size distributions of young squid were skewed toward larger animals. We concluded that the size distribution of young squid was not simply the result of developmental temperature. Other factors, including the time distribution of egg deposition, vertical distribution of newly hatched squid, predation pressure and planktonic dispersal probably affect the measured size distribution of young squid.

Fields (1965) reported the polychaete *Capitella ovincola* from the intermediate jelly of *L. opalescens* egg strings. *Capitella hermaphrodita* lives and reproduces in the jelly of *Loligo vulgaris* egg strings (von Boletsky and Dohle, 1967). We did not observe any polychaete or other macroscopic commensal in *L. pealei* egg strings.

Chromatophores become evident at stage 26, the beginning of phase 3 (Arnold, 1965). Newly hatched squid have active chromatophores of three distinct colors: red, brown and light green which become pink, reddish brown and light green, respectively, when expanded. The latter becomes orange (yellow expanded) within a few days after hatching. Two large reddish brown chromatophores are located on the dorsal mantle surface between the fins. These remain expanded for at least a week after hatching (the maximum survivorship in our experiments).

In the laboratory under all conditions of illumination, newly hatched squid expend considerable amounts of energy to remain at or near the water surface. Repeated failures to collect numbers of young squid with surface plankton nets suggest that this behavior is a laboratory artifact.

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SUMMARY

1. *Loligo pealei* embryos were readily maintained in flowing sea water between 12.0 and 23.0° C. They were observed and staged according to Arnold's (1965) description of normal embryonic development. Developmental stage *vs.* time plots fell into three groups with sea water temperature ranges of 12.0–18.0° C, 15.5–21.3° C and 21.5–23.0° C and mean total developmental times of 642, 445 and 257 hr, respectively.

2. The rate of development appeared to be directly related to sea water temperature and could be modified at any stage by altering sea water temperature. Extrapolations of this relationship are possible, and practical limits of its extension are discussed.

3. Development apparently consisted of four phases; the first three each requiring a specific proportion of the total developmental time independent of tempera-

ture and the fourth (hatching), requiring a specific time interval independent of temperature. Development to stage 12 was non-linear and required 13% of the total time. Linear development over stages 12 to 26 required 51% of the total time. Stages 26 to 29 also developed linearly, but required 22% of the total time. In each group, development from stage 29 to stage 30 (hatching) required approximately 52 hr, apparently independent of temperature. Functonal distinctions are suggested for these developmental phases.

4. Approximate time of first hatch could be predicted from the sea water temperature at egg deposition or from an observed time interval between established stages during development.

5. Artificial size distributions constructed from the developmental data and observed sea water temperatures differed markedly from measured size distribution of young squid. Factors other than temperature probably affect the measured size distributon of young squid.

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PARTICLE FEEDING IN NATURAL POPULATIONS OF THREE MARINE DEMOSPONGES

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The precise ecological roles of sponges remain undefined primarily because natural food materials have never been reliably determined for any member of the phylum. Early demonstrations of particle retention by sponges using non-nutritive materials (India ink, carmine, *etc.*) indicated that particle capture is accomplished by either amoebocytes or choanocytes, depending upon particle size and species of sponge studied. Both van Trigt (1919) and van Weel (1949), in providing nutritive but non-natural material to fresh-water spongillids, found that the site of particle capture was dependent on particle size. Large particles over 50 μ, unable to transverse ostia, were picked up by the surface epithelium; particles 5–50 μ were phagocytosed by archaeocytes and collencytes lining the inhalant passages; and particles smaller than 5 μ passed the prosopyles and were captured by choanocytes at the cell base or collar. Pourbaix's observations (1931, 1932a, 1932b, 1933a, 1933b) on living spongillids and on histological specimens of marine demosponges coincided with these findings and further indicated that capture sites may vary somewhat from species to species.

More recent works have been less conclusive and have cast doubt on the validity of the earlier works. Kilian (1952, 1964), in carrying out a detailed study of particle uptake using albumin-India ink and fresh-water spongillids, clearly substantiated the observations on van Trigt (1919) and van Weel (1949) in detail. His attempts to obtain lasting cultures of spongillids with live bacteria, however, failed. Because of this and his inability to demonstrate bacteria in intracellular food vacuoles, he concluded that bacteria could not serve as natural food materials for fresh-water sponges. Rasmont (1961, 1968), however, was able to obtain growth and maturation of spongillids on a diet of cleaned dead bacteria. His co-worker, I. Schmidt (Rasmont, 1968) observed that bacteria capture was effected by choanocytes. Simpson (1963, 1968) in attempting to substantiate the earlier observations of Pourbaix, was unable to find evidence of extra-nuclear DNA in choanocytes of the marine demosponge *Microciona prolifera*. He concluded that choanocytes probably do not serve as important sites of natural particle capture in this species. Works by Claus, Madri and Kunen (1967) and Madri, Claus, Kunen and Moss (1967), reporting to have demonstrated bacterial "removal" by *M. prolifera* held for long periods in immense cultures of *Escherichia coli*, have simply shown that *E. coli* numbers decrease in closed aquaria containing large masses of the proven bacteriostatic sponge *M. prolifera*. Capture and retention of bacteria by the sponges was not directly investigated, nor was it likely to have occurred in their experimental set-up.

All of the above works lack applicability for ecological analysis of marine demosponges in that they were qualitative, were performed with non-natural food materials usually under adverse laboratory conditions, or were based on work with the specialized group of fresh-water sponges. The present study is an attempt to resolve some of the contradictions in these observations and to provide a firm qualitative and quantitative assessment of the natural food materials of three species of marine demosponges.

Materials and Methods

Three species of Demospongiae were selected for investigation on the bases of (1) abundance and importance in the local ecosystem, (2) taxonomic distance between species and (3) morphology, utilizing specimens with single large oscula for ease of water sample collection. Each of the species selected is a dominant or important member of the lush Porifera fauna of the north coast of Jamaica. The work was carried out at the UWI-SUNY Marine Laboratory at Discovery Bay, Jamaica.

Tethya crypta (de Laubenfels, 1949, as *Cryptotethya*), S. C. Tetractinomorpha O. Hadromerida, is common throughout the eastern shallow protected areas of Discovery Bay, Jamaica. Within this habitat, the species is restricted to depths of −1 to −6 meters. All investigations of this species were made on a locally dense population situated at −3 m. The other two species, *Verongia gigantea* (Hyatt, 1875), S. C. Ceractinomorpha O. Dictyoceratida, and *Mycale* sp., S. C. Ceractinomorpha O. Poecilosclerida, comprise major components of the sponge-dominated fauna of the deep outer coral reefs. (The *Mycale* here, presently without a valid specific name, is that described by de Laubenfels, 1936, p. 116, but erroneously referred to as *M. angulosa*.) Although these two species coexist over a considerable portion of their ranges, −24 to −52 and −15 to −55 m, respectively, the center of abundance of *Verongia* on the deep fore reef is significantly deeper (−43 m) than that of *Mycale* at the junction of the fore-reef slope and the deep fore reef (−33 m) (reef nomenclature after Goreau and Wells, 1967). These two species show partial physical niche separation in this relatively nannoplankton-poor habitat.

In a typical higher demosponge, seawater streams slowly into the extensive inhalant surfaces, traverses a series of three successively finer, discrete filtration systems (1, dermal membrane, 2, inhalant canals and prosopyles, 3, choanocyte collars; van Gansen, 1960; Jørgensen, 1966), is re-collected by a system of converging exhalant canals and is ejected at high velocity from the vicinity of the sponge through the large exhalant osculum (or oscula). Essentially all exchanges of materials (food, respiratory gases, wastes, *etc.*) taking place between the specimen and the environment occur during transit of water through the canal systems. To investigate the natural exchanges of particulate organic material occurring in field populations of the three species noted above, samples of water were collected *in situ* from near the inhalant surfaces (ambient water) and from the oscular stream (exhalant water). The samples were individually analyzed for plankton by direct microscopy and for total particulate organic carbon (POC) by chemical analysis. The comparison of ambient and exhalant samples provides direct assessment of the rates of filtration of plankton particles by type and size, as well as an overall assessment of the natural diets of the three species studied.

Water samples for direct microscopic analysis of plankton were collected during summer and winter in clean 25 ml polyethylene syringes. Exhalant water samples were obtained by carefully inserting the capped syringes into the exhalant stream below the margin of the osculum, taking care not to contact the specimen. The caps were removed while in the stream, the syringes slowly filled and the caps replaced while still in the stream. Ambient samples were taken near the inhalant surface of the same specimens immediately following collection of the exhalant samples. Within one hour of collection, 15 ml aliquots of each of the samples were filtered through 25 mm 0.22 μ Millipore type GS membrane filters at a suction of 0.3 atm or less (Holmes and Anderson, 1963). The filters were fixed in formalin fumes for 30 minutes, air dried, and stained in a membrane-filtered solution of 1% erythrosin-phenol for 1 hour at 60° C (Jannasch and Jones, 1959; Kriss, 1963). They were then rinsed in distilled water, air dried, and mounted in balsam under cover slips on microscope slides. A total of 30 such ambient/exhalant pairs of samples were collected and examined by direct microscopy.

Organic (red-stained) particles on each filter, within the size range able to pass the 50 μ diameter pores of the dermal membrane of these sponges (*i.e.*, having no more than one major axis greater than 50 μ), were recognized as 4 major fractions by size and morphology: (1) bacteria, (2) unarmored cells, (3) armored cells (fungi, diatoms, dinoflagellates, coccolithophores, *etc.*) and (4) detritus. The term "detritus" is used here in the narrow sense (see Bakus, 1969) to include only discrete, visibly resolvable, organic particles which are obviously non-living and which consist primarily of cellular debris and planar flakes. This is in contrast to the general, nonspecific, operational definition (see Jørgensen, 1966) for all supposed non-living material retained by glass fiber filters.

Two size classes of bacteria were recognized and enumerated separately in 5 randomly selected fields of each filter under oil immersion (1600 ×) for a total area scanned of 0.059 mm^2 or effectively 0.00356 ml of the sample. Although bacteria were small, 0.3–0.8 μ in greatest dimension, little or no practical difficulty was encountered in recognition due to the superior surface characteristics of the 0.22 μ membranes (in contrast to the previous 0.45 μ standard) and the general lack of similar-sized inorganic particles in these waters. Clumping of bacteria was found to be very rare at these concentrations and offered no problems to enumeration. Length and width of 25 bacteria of each of the 2 size classes were estimated against a 0.4 μ micrometer scale to $\pm$ 0.05 μ. Particle volume of the 2 sizes was calculated assuming a regular ovoid form.

All larger particle fractions (2–5 μ) were enumerated in a single continuous scan across a major diameter of each filter under high-dry 44 × objective (700 ×), covering a total area of 4.68 mm^2 or effectively 0.28 ml of the sample. After suitable familiarization with the spectrum of particle shapes of the local plankton, a system of 25 shape categories was developed for armored plankton groups. A relationship between length and particle volume was determined for each shape category (*e.g.*, volume of dinoflagellate $A = 0.335\ L^3$; *etc.*), based on length, breadth, and width measurements of 25 particles of each category and approximations to regular geometric shapes (for similar methods see: Jørgensen, 1966, Sec. 2.IIII; Mullin, Sloan and Eppley, 1966; and Strathmann, 1967). In scanning each filter, the length of every particle encountered was measured and its volume approximated on the basis of its shape category. Because of the poor quality of preservation, plasma volumes of diatoms could not be readily determined, and thus only cell volumes were obtained (see Strathmann, 1967, for a discussion of significance).

While the shape of armored cells is reliably maintained on filters, and the direct geometric procedure provides a suitable means for volume approximation, it is not suitable for unarmored cells. The diameter of the flattened ghosts of unarmored cells retained on the filters bears an unknown relationship to the volume of the original cell. For procedural reasons I have employed the assumption that the surface area of the ghost (both sides) reflects the total surface area of the flattened original cell pellicle and thus original cell volume = approximately $0.185 \times$ ghost diameter^3. If these cells do not spread laterally (*i.e.,* maintain constant cell volume as they encounter the filter surface), but instead simply collapse as the cytoplasm flows through the rupture at the point of first contact with the filter, then contraction of the pellicle would be severe, and the true cell volume may be 2 or 3 × that estimated by the assumptions adopted here.

The organic carbon content of the 4 microscopically resolvable fractions (MPOC) was estimated for each sample from the above determined particle volume and from the relationships of carbon to volume (C/V ratios from other works). A general C/V ratio of 0.10 was employed for the bacterial fraction (Oppenheimer and Jannasch, 1962, use 0.05; ZoBell, 1963, uses 0.10; Jørgensen, 1966, uses 0.11). The C/V ratios applied to plankton cell fractions were adopted from Strathmann's (1967) work on analysis of phytoplankton cultures:

$$\text{for diatoms:}\quad \log C = -0.422 + 0.758\ (\log V);$$
$$\text{for all other cells:}\quad \log C = -0.460 + 0.866\ (\log V).$$

These size-dependent ratios were applied to each and every armored plankton cell encountered on each filter. Although an appropriate C/V ratio is unavailable for detrital material, a not unreasonable C/V ratio of 0.25 has been employed for this fraction on the basis of its generally accepted high cellulose content.

The procedures of enumeration provided mean raw counts of 141 and 406 bacteria per ambient sample of outer reef and bay waters, respectively. A total of over 14,500 bacteria were directly counted and assigned to class size. The high-dry scan covered an effective area of 87 fields and produced mean raw counts of the 3 fractions enumerated of 105 and 144 particles per ambient sample of outer reef and bay water, respectively. Size measurements and calculations of volumes and POC were made on a total of over 6500 individual particles of fractions 2–4. For individual fractions, mean counts for outer reef and bay water, respectively, were: unarmored cells, 64, 72; armored cells, 31, 47; detritus, 10, 17. Numbers of bacteria and totals for the 3 other fractions are considered to be highly dependable, within 20% of the true value for each sample. Counts of the rarer fractions—detritus and armored cells—are considered to be inaccurate because of the low numbers of cells, and may vary from the true value by a factor of 2 for any single sample.

Final calculations of POC content include not only true variations in the abundance of each fraction resulting from inherent patchiness and variations in seasonal abundance, but also include variations resulting from enumeration procedures, size measurements, and utilization of empirically derived C/V ratios. A reasonably accurate determination of the artificial variation attributable to handling and other procedures can only be obtained through replicate analysis of individual samples—which was not carried out.

Estimates of bacterial POC of any single sample are considered to be within $\frac{1}{3}$ to 3 × the true value, most of this attributable to possible error of size estimation. The POC values for unarmored cells may be $\frac{1}{10}$ to 3 × the true value for any given sample, due mainly to the assumptions of size and C/V ratio. Volume determination of armored and detritus particles is considered highly accurate, since size and shape are maintained throughout procedures. The POC values of these 2 fractions are still considered only to be within $\frac{1}{4}$ to 4 × the true value for any given sample primarily due to relatively large probable errors resulting from the random occurrence of large individual particles.

Although error of the estimate of POC content for any single fraction in any single sample is admittedly high, statistical treatment of adequate sample numbers compensates for sampling errors in determination of mean values with high accuracy (standard errors provided with data). Inasmuch as measurement and conversion errors are applied uniformly to both ambient and exhalant samples, the differences between samples—that is, effective filtration rates for specific particle fractions—are relatively independent of even these errors, as indicated by narrow confidence intervals supplied with data. These direct methods still remain the best available for quantification of plankton standing stock (Wood, 1968a).

In Jamaican waters zooplankton was essentially restricted to near-surface layers, the waters bounding the reef or bay bottom remained effectively free of larger particulate materials. Phytoplankton exceeding the size limits of the dermal pores of sponges (2 dimensions greater than 50 μ) were also scarce in bottom boundary waters—estimated from filter abundance to amount to less than 5% of the total plankton standing stock. No particles larger than 50 μ in greatest dimension were encountered in exhalant water samples. In all samples analyzed, the 4 particle fractions enumerated included at least 99.8% of the resolvable particulate material able to pass the dermal pores (by particle volume) and 95% of the total plankton. The aperture of the syringe collector was of sufficient diameter (2 mm) to preclude selective exclusion of particulate material present.

Water samples for chemical determination of particulate organic carbon (CPOC) were collected in cleaned 6-liter polyethylene bags fitted with 2–5 cm diameter stoppered closures. A stoppered bag was suspended by tripod above the specimen and oriented with the closure inserted into the oscular opening. The stopper was then removed and the bag was inflated by the low pressure of the exhalant stream of the specimen. Collection time for a 6-liter exhalant sample varied from 30 seconds or less with large specimens of *Verongia* to 15 minutes for small *Mycale* and *Tethya*. An ambient water sample was taken from the vicinity of the inhalant surface of the same specimen in an identical container during collection of every exhalant sample. When fully inflated, the exhalant sample bag was capped while still in the exhalant stream and returned to the laboratory. After the bags were rinsed in distilled water, the 6-liter water samples were filtered through pre-combusted 4.25 cm Reeve Angel #934AH glass fiber filters (tested as superior in particle retention to standard Whatman type GF/C), and analyzed for POC following the "wet ashing" acid dichromate method outlined by Strickland and Parsons (1965). Extinction was measured on a Bausch and Lomb Spectronic 20 and POC calculated to ± 1 mgC/m^3.

Eleven such ambient/exhalant paired samples were collected and analyzed for CPOC. Aliquots of 8 of these pairs of samples were also analyzed by direct microscopic methods to determine differences in performance, if any, between the plastic bag and syringe collectors. Statistical tests of total particle volume and cell numbers of larger plankton fractions indicate there was no significant difference between ambient water samples collected with syringes or bags $(0.10 < P < 0.90$, Wilcoxan two sample statistic).

The basic difference in operation of the two collectors does, however, allow expectation of slight differences in exhalant samples. The syringe is basically a rigid, wiped-piston collector, and thus ambient water is potentially able to move by the rubber seal to compensate for compression of small gas bubbles within the syringe neck during descent. The action of drawing back the piston during sample collection also potentially allows some ambient water and thus particulate material to enter the barrel from the piston end. Exhalant water samples collected by syringe could be expected to be slightly contaminated with ambient water and indicate lower filtration rates than the bag samples, with an expected bias to small particle sizes. It appears that this does, in fact, occur, although the net contamination is slight (to be discussed below). Comparison of bag samples with samples collected in pre-cleaned glass-stoppered bottles indicated that the polyethylene itself contributed no detectable carbon to the samples within the limits of resolution of the analytical procedures.

The type of water samples available in this study—samples of seawater collected *in situ* before and after true single-pass filtrations—has not previously been employed in analysis of particle selection in any filter feeding organism. This study is, therefore, entirely free of the usual criticisms encountered in laboratory studies: (1) unknown feedback influences due to recycling of media in closed systems (*e.g.*, Jørgensen, 1949); (2) contamination of post-filtration samples with unknown quantities of unfiltered media (*e.g.*, Haven and Morales-Alamo, 1970); and (3) the universal problem of indeterminate influences of non-natural laboratory conditions.

RESULTS

Available particulate organic materials

The distribution of particulate materials potentially available as food for the sponges studied here are presented in Table I in terms of particle numbers, particle volume, and organic carbon (MPOC, calculated). The data effectively represent means of approximately equal numbers of samples collected in late summer (September–October 1969) and late winter (Jaunary–March 1970), periods of maximum and minimum temperatures. All bay samples were taken from a restricted area at −3 m; outer reef samples were collected over the range −15 to

−52 m. All ambient samples analyzed by direct microscopy (30 syringe samples and 8 plastic bag samples) are incorporated. The relatively richer shallow bay waters contain approximately 1.7 × the particulate organic material (in volume or carbon) found in the clear outer reef waters. Water within the bay also maintains significantly higher standing stock of each particle fraction except detritus for which differences between habitats are not statistically significant. Significant seasonal variations in abundance of specific plankton fractions do oocur but are beyond the scope of this report.

TABLE I

Available particulate material from analysis of ambient water samples

Particulate fraction	Bay (11 samples)	Outer reef (27 samples)	Stat. signif. P
Particle concentration—number/ml ± s.e.			
1 Bacteria	114,000 ± 21,600	39,740 ± 4,340	< 0.01
2 Unarmored cells	275.7 ± 32.7	227.3 ± 26.1	NS
3 Armored cells	166.7 ± 17.3	109.5 ± 11.6	< 0.01
4 Detritus	60.2 ± 9.3	35.7 ± 5.7	< 0.05
All non-bacterial particles	516.5 ± 47.0	374.8 ± 34.2	< 0.05
Particle volume—$10^3\mu^3$/ml ± s.e.			
1 Bacteria	5.72 ± 1.20	1.96 ± 0.21	< 0.01
2 Unarmored cells	42.67 ± 6.63	28.02 ± 3.41	< 0.05
3 Armored cells	12.03 ± 1.52	4.59 ± 0.56	< 0.01
4 Detritus	1.72 ± 0.39	1.03 ± 0.17	NS
Total all particles	62.24 ± 6.57	35.62 ± 3.51	< 0.01
Calculated organic carbon—mg/m^3 ($= 10^{-9}$g/ml) ± s.e.			
1 Bacteria	0.571 ± 0.120	0.196 ± 0.021	< 0.01
2 Unarmored cells	6.74 ± 0.89	4.48 ± 0.51	< 0.05
3 Armored cells	1.60 ± 0.149	0.706 ± 0.081	< 0.01
4 Detritus	0.408 ± 0.089	0.258 ± 0.042	NS
Total all particles (MPOC)	9.336 ± 0.906	5.565 ± 0.530	< 0.01
Total range	(5.53 – 14.99)	(1.49 — 13.0)	
Chemically determined total POC—mg/m^3 ± s.e.	(6 samples)	(5 samples)	
Total CPOC	85.95 ± 6.61	63.88 ± 3.27	< 0.01
Unresolvable POC***	75.70 ± 12.75**	55.00 ± 2.21	< 0.025

* Wilcoxan (Mann-Whitney) two sample statistic.

** Only 3 of the 6 samples were directly analyzed by microscopy.

*** Calculated as total CPOC minus MPOC.

Bacteria (fraction 1), while present in moderate numbers, comprise only 4–10% of the biomass of available directly resolvable particulate material. Unarmored cells (fraction 2), 5–50 μ in diameter, constitute the largest single resolvable fraction (70–80% of total) in both habitats in all seasons. These cells are recognized on filters as flattened cell ghosts with small central nuclei surrounded by mitochondrial particles. Recognizable chloroplasts are lacking. The fraction undoubtedly includes the naked flagellates, generally the major element of plankton biomass of tropical waters (Hulburt, Ryther, and Guillard, 1960; Bernard, 1963; Mullin, 1965; Wood, 1968c).

The armored cells (fraction 3) constitute 13–20% of the biomass of available organic material. Two small classes of heavily armored particles, 2 × 2 μ and 3 × 4 μ, are present in high numerical abundance ($10^4 - 10^5$ cells/liter), but account for only 5–20% of the total armored cell fraction. These are similar to those reported in the Sargasso Sea (Hulburt, Ryther and Guillard, 1960) and the Mediterranean Sea (Bernard, 1963), and probably include several species of fungi and resting capsules of blue-green algae. Diatoms, dinoflagellates and coccolithophores alternate in abundance seasonally but maintain a relatively constant total. At least seven species of diatoms among the genera *Pinnularia, Nitzschia, Coscinodiscus* and *Licmophora* are present in both habitats, with greatest dimension of 5–100 μ. Dinoflagellates and coccolithophores, varying from 5–20 μ in greatest dimension, are dominated by representatives of *Gymnodinium* and *Oxytoxum* (from Wood, 1968b). No single armored species is overwhelmingly dominant in any single sample.

Detrital particles (fraction 4), including skeletal debris of plankton and flat flake-like structures, account for only 3% of the total resolvable material by volume and 5–10% by calculated carbon.

Chemical analysis of total particulate organic carbon (CPOC) was carried out only on late winter samples. Both bay and outer reef waters contain POC values within the range reported for open waters of the Gulf of Mexico by Fredericks and Sackett (1970) and are considered to be typical, nutritionally-poor tropical waters. The results of the two methods of analysis indicate that fully 86–88% of available carbon of these samples cannot be accounted for by microscopically detectable particulate fractions—including detrital material. By convention such differences between total CPOC and plankton POC, normally encountered in analysis of seawater throughout the world, are attributed to detrital material (Jørgensen, 1966). In the samples of Jamaican waters studied here, detrital material is not sufficiently abundant to account for this discrepancy, even if a C/V ratio of 1.0 is employed for this fraction. On the basis of the available information (and other evidence to be discussed below) it appears that the major portion of the functionally particulate organic carbon in Jamaican water is present as material unresolvable by direct microscopy (URPOC).

Retention rates by particle type and size

The per cent efficiency of retention,

$$\frac{\text{ambient-exhalant}}{\text{ambient}} \times 100$$

is shown in Table II and Figure 1 for each particle fraction for each species of demosponge investigated. Means and 95% confidence limits of the means were approximated using arcsin percentage transformation and student's t distribution (Owen, 1962). Levels of significance of differences in retention between species of sponges for given particle fractions, and conversely between particle fractions for a given species of sponge, were calculated using the Wilcoxan (Mann-Whitney) two sample statistic (Owen, 1962).

Bacteria were retained by all three species at high efficiency-mean of the three species is 96.1% by number of particles and 96.4% by calculated volume or carbon (rates of retention of carbon and volume are identical since a single, size-independent C/V ratio was employed for this fraction). Retention differences between species are insignificant for the complete fraction, as well as for and between the smaller ($0.028\ \mu^3$) and larger ($0.151\ \mu^3$) size classes of bacteria ($P > 0.10$ in all cases). Within the ranges of bacterial concentration encountered, retention rates were found to be independent of abundance.

TABLE II

Retention efficiencies of natural particulate organic fractions by three sponges

Fraction	Mean % retention (95% confidence interval of the mean)		
	M. sp.	*V. gigantea*	*T. crypta*
Bacteria	10 samples	7 samples	11 samples
Bacteria			
By number	96.9 (95.0– 98.4)	94.8 (91.3– 97.5)	96.5 (94.7– 98.0)
By volume	97.6 (95.6– 98.9)	95.1 (90.2– 98.4)	96.2 (94.0– 98.0)
Other fractions	14 samples	13 samples	11 samples
Unarmored cells			
By number	80.4 (73.9– 86.2)	82.5 (76.8– 87.6)	94.4 (91.0– 97.0)
By volume	88.2 (83.2– 92.3)	88.4 (83.9– 92.2)	91.7 (82.4– 97.7)
Armored cells			
By number	41.2 (30.3– 52.5)	38.7 (19.3– 60.2)	66.0 (53.4– 77.5)
By volume	64.5 (44.9– 81.8)	77.0 (61.7– 89.4)	80.1 (64.5– 92.0)
Detritus			
By number	−172.3 (+67.9– −412.5)	−186.4 (+32.2– −405.0)	−148.5 (−2.7– −294.3)
By volume	−51.7 (+35.7– −140.1)	−113.5 (−11.8– −215.5)	−82.8 (+12.8– −178.4)

During a one-month period in mid-winter (16 January–17 February 1970), bacterial retention of both outer reef species, *Mycale* and *Verongia*, dropped far below the normally high 96% levels, while retention rate of other plankton fractions was unaffected (Fig. 2). This partial failure of only the bacterial capture system of both outer reef species, while retention of the bay species *Tethya* remained unchanged, suggests that the cause was a local shift in the composition of the bacterial fauna. The lack of change either in morphology of bacteria or abundance ratios of the 2 size classes suggests that the species composition of bacteria did not undergo a major shift at this time. The action of normal winter storms during and preceding this period had, however, caused heavy general mortality of the outer reef sponge fauna, leaving considerable numbers of damaged and decomposing sponges throughout the fore-reef slope. Bacteria, certainly utilizing these sponges as a nutritional substrate, may have incorporated significant

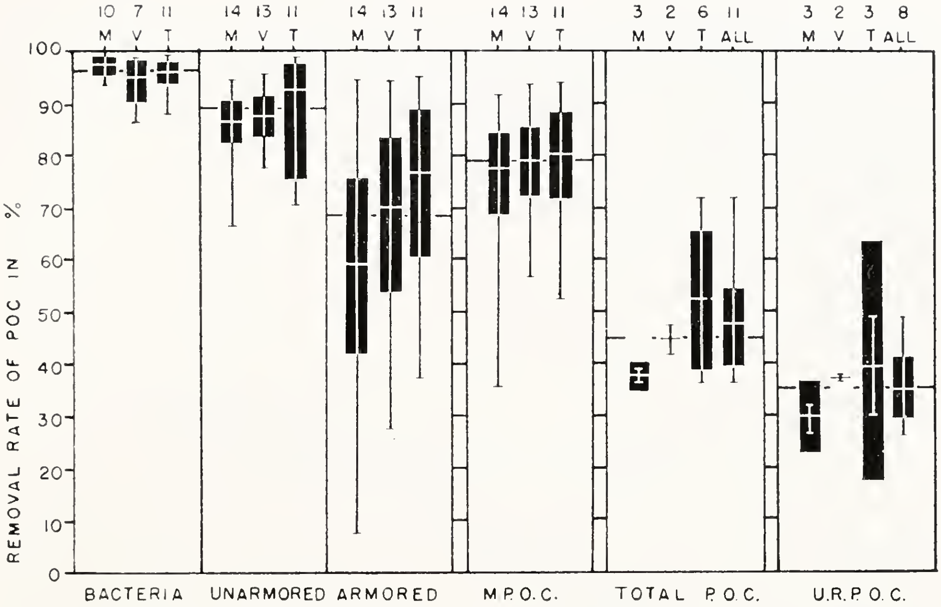

FIGURE 1. Per cent removal rates for various POC sources. Number of samples and sponge species are designated above the graph: M = *Mycale* sp.; V = *Verongia gigantea;* and T = *Tethya crypta.* Total range of samples is represented by the vertical line, mean by the horizontal (white) line, and 95% confidence interval of the mean by the solid bar (where calculable). The mean of the three sponges is shown as the broken horizontal line across each fraction interval. Range, mean and confidence intervals for all available samples of total POC and URPOC are shown to the right of these intervals.

amounts of distinctive sponge sterols (Bergmann, 1949) in surface membranes, which could have elicited rejection of these bacteria by the healthy, filtering sponges.

As has been previously indicated, the retention rates for bacteria may be slightly underestimated above due to partial failure of the syringe sample collector.

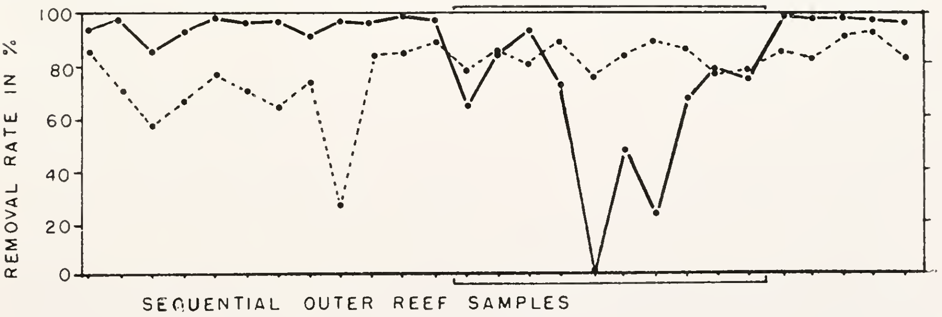

FIGURE 2. Mid-winter failure of bacterial retention by both outer reef sponges. The removal rates of bacterial POC (unbroken line) and all other MPOC (broken line) are shown for sequential water samples collected on the outer reef. Failure of bacterial retention (within brackets) occurred in both species, while retention of other fractions remained at normal levels.

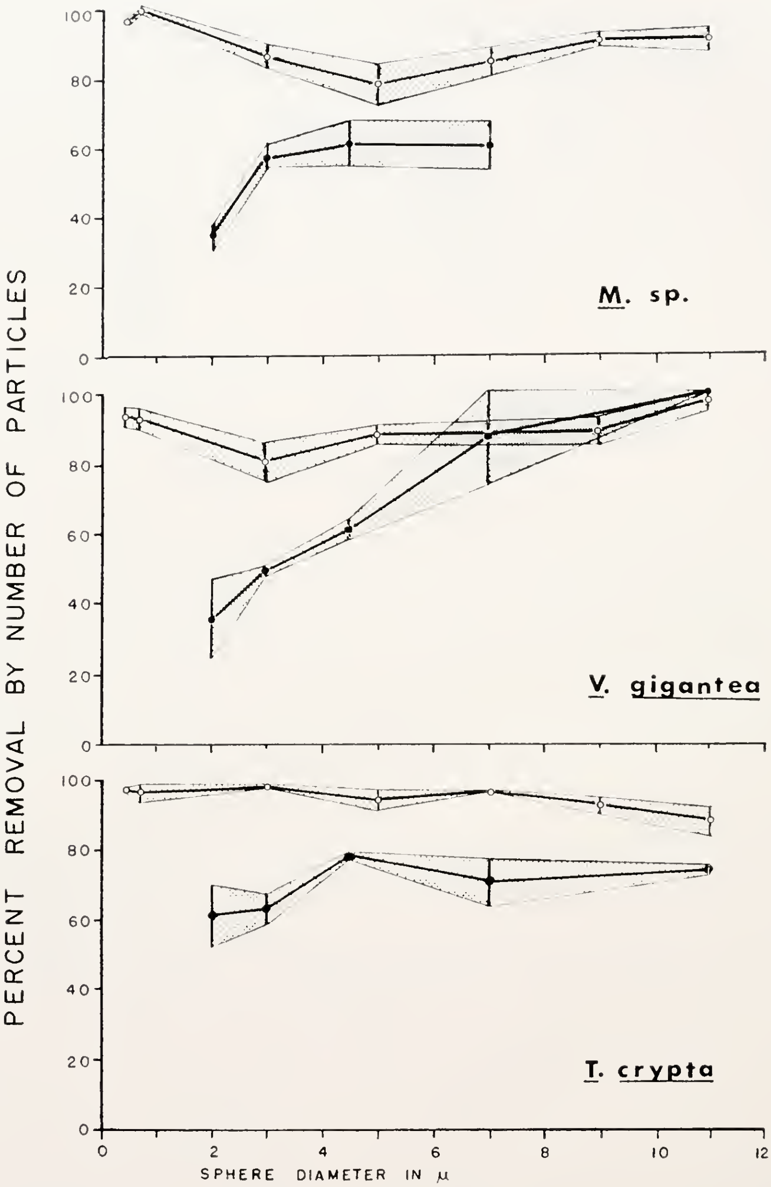

FIGURE 3. Relationship of particle size to retention rates for resolvable plankton fractions. Particles within fractions have been grouped by volume expressed here as diameter of sphere with equal volume. All winter and all summer samples have been grouped to provide adequate numbers of particles (> 30) within each size interval resulting in only 2 points for each grouping. Armored cells are shown in the lower block-area of each species, unarmored cells in upper. The unarmored fraction has been extended to the left, connecting with the two bacterial size classes.

Samples collected by plastic bags, which are free of the operational bias of syringes, show a mean retention rate of 98.1% by volume or carbon (8 samples). The probable contamination of exhalant samples by ambient water passing the plunger seal amounted to only approximately 1.7% of the sample volume.

Unarmored cells (fraction 2) are retained by all three species at high efficiency-means for the three species are 86.5% by numbers of cells and 89.3% by calculated cell carbon (Table II, Fig. 1). Retention rate by cell carbon for this fraction is significantly higher ($P << 0.001$) in *Tethya* than in the other two species, which do not differ ($P > 0.10$). The retention rate for unarmored cells by carbon is not significantly different from that of bacteria for *Tethya* ($P > 0.10$), while *Mycale* and *Verongia* both retain unarmored cells at significantly lower efficiencies than bacteria ($P < 0.01$). All three species show little evidence of size selectivity within the unarmored fraction (Fig. 3).

Armored cells (fraction 3) are retained at moderate rates by these three sponges—mean for the three species is 48.7% by cell numbers and 68.7% by cell carbon (Table II, Fig. 1). Due to high variability of individual samples (low particle counts) and to slightly different composition of the armored cell fractions in the two habitats, significant differences between the three sponges vary with analysis. By cell numbers, *Tethya* retains armored cells at signficantly higher rates ($P < 0.01$) than the other two species which do not differ ($P > 0.10$). By volume or cell carbon, a more valid estimate of potential food value, no significant differences are found between the three species in retention of this fraction. By carbon, all three species retain the armored fraction at significantly lower rates than the unarmored fraction ($P < 0.01$ in each case) and bacterial fraction ($P < 0.001$ in each case).

Within the armored fraction definite evidence in size selectivity is shown by two of the three species (Fig. 3). Smaller 2 μ diameter armored cells, within the size range of the prosopyle openings serving the flagellated chambers, are retained at very low efficiencies—20–45%—by both *Mycale* and *Verongia*. *Mycale* and *Tethya* exhibit a fairly constant rate of retention in larger armored particles, while *Verongia* retains larger cells with increasing efficiency—nearing 100% retention in larger effective cell sizes. Possible mechanisms for these differences will be discussed below.

Each of the three species of sponge exhibits a net production of detrital material (fraction 4) (Table II). Exhalant water samples contain approximately 2.7 × the number particles and 1.8 × the carbon content (calculated) of corresponding ambient water samples (means of the three species). Because of extreme variability in abundance of ambient detrital material, and the variability in production/retention values of individual sample pairs, confidence in the level of production of this material is very low. Due to the same variability, no statistical differences were found between the three species. In each case, the production of this fraction contrasts sufficiently with the retention of the other three fractions to obviate statistical demonsration of differences. The detrital material produced probably consists of incompletely digested cellulose walls and cell debris from the three major fractions of plankton particles. As there is no reliable way to distinguish newly produced detrital material from that taken in with ambient water, the extent to which available detrital particles are utilized for food by these sponges cannot be determined with the methods employed.

All microscopically resolvable particulate material able to pass the 50 μ dermal pores is retained at fairly high rates by all three species (Table II, Fig. 1) —mean of the three species is 79.0% by calculated carbon content. This data includes the 4 fractions treated above and a minor component of miscellaneous particles not assignable to those fractions (filamentous blue-green algae, small fungi, *etc.*) The retention rates by numbers of particles (omitting bacteria) are low (mean = 41.9% for the three sponges), due primarily to the net production of large numbers of detrital particles which do not greatly lower volume or carbon values. The overall rates of retention for all particulate material do not differ significantly between the species ($P > 0.10$ in each case).

Table III

The net diets of three sponges in terms of POC removed from each particulate fraction per unit of seawater filtered

Fraction	POC source—mg/m^3—s.e. (number of samples)			% of total POC retained (mean of three species)
	M. sp.	*V. gigantea*	*T. crypta*	
Bacteria	0.171 ± 0.037 (10)	0.137 ± 0.035 (7)	0.546 ± 0.116 (11)	0.89
Unarmored cells	3.77 ± 0.66 (14)	4.18 ± 0.75 (13)	6.07 ± 0.78 (11)	16.2
Armored cells	0.386 ± 0.078 (14)	0.622 ± 0.135 (13)	1.19 ± 0.16 (11)	2.4
(miscellaneous)	*	*	0.008 ± 0.002 (11)	0.007
Detritus	−0.042 ± 0.077 (14)	−0.117 ± 0.093 (13)	−0.382 ± 0.197 (11)	—
Total MPOC	4.29 ± 0.73 (14)	4.82 ± 0.77 (13)	7.43 ± 0.86 (11)	19.5
URPOC	15.81 ± 1.06 (3)	21.28 ± ** (2)	32.28 ± 6.30 (3)	80.5
Total POC	20.1	26.1	39.7	100.0

* Negligible.
** Only 2 samples available.

Chemically determined total CPOC

Comparison of CPOC content of the 11 ambient/exhalant pairs of large water samples indicates that total "particulate" organic carbon is retained by these sponges at low efficiencies (Fig. 1)—the mean of the three species is only 44.7%. The three sponges show no statistically valid differences, as expected from the small numbers of samples. The mean retention rate of all 11 paired samples is 47.7%, with a reasonably small 95% confidence interval for the mean (Fig. 1).

It has been suggested above that the major portion of chemically measurable POC (*i.e.,* retained on the glass fiber filters) in ambient water samples is not accountable as microscopically detectable plankton particles, but apparently exists as non-discrete non-resolvable material. The filtration rates derived for the 4 microscopically resolvable fractions and for total CPOC are consistent with this assumption, and provide supplementary evidence for the existence of the URPOC fraction (to be discussed in detail below).

The rates of retention of the preponderant fraction of available carbon as URPOC material were obtained from 8 of the large sample pairs collected in late winter. The calculated amounts of MPOC were subtracted from CPOC, providing estimates of URPOC of ambient and exhalant samples. Retention rates of URPOC are low (Fig. 2), mean of the three species is 35.2%. As only 2 or 3 sample pairs are available for each species, differences between species cannot be statistically analyzed. The mean retention of URPOC for all 8 samples is 35.0% with a 95% confidence interval of 29.4–40.8%. The differences between retention of this URPOC material and all MPOC fractions is significant ($P < 0.001$).

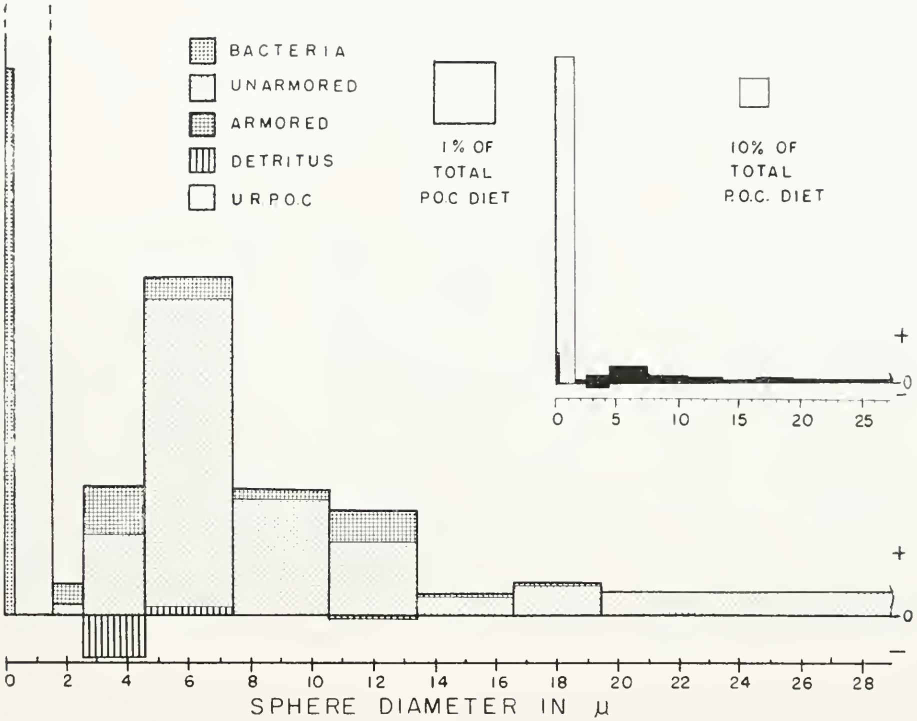

FIGURE 4. Mean size distribution of the dietary POC for three marine Demospongiae. All particles surveyed in the samples of each species have been grouped by volume interval (expressed here as diameter of sphere with volume equal to interval mid-point). Total POC content of each size interval was summed for each fraction and means of the three species are expressed as area of the interval block. Net retention and production are respectively shown above (+) and below (−) the zero line. URPOC is restricted to the interval between 1.5 and 0 μ effective size (see discussion). The total contributions of URPOC and MPOC are shown in the reduced inset (upper right).

Net POC diet of sponges

The estimated mean sources of dietary POC for the three sponges over the entire year (all samples) is presented in Table III. Because of its relatively high abundance, the URPOC fraction provides by far the major carbon source. The three plankton fractions, although retained at high efficiencies by all three species, contribute a minor proportion, due to their relatively low concentrations in both habitats.

The size distribution of retained, and presumably utilized, POC within each fraction has been analyzed for each species, the mean of the three is provided in Figure 4. Particle size is represented on the abscissa as diameter of a sphere of equal volume. Particles are grouped in intervals of sphere diameter of: 1.5–2.5 μ, 2.5–4.5 μ, 4.5–7.5 μ, *etc.* Calculated POC is represented by area within each size interval for each fraction. Most retained MPOC consists of particles between 8.2 and 1,290 μ^3 in volume, or equal to spheres of 2.5 to 13.5 μ diameter. The reasonable restriction of URPOC to material less than 1.5 μ in effective diameter (to be substantiated below) renders the overall size distribution of dietary POC conspicuously bimodal (Fig. 4, insert).

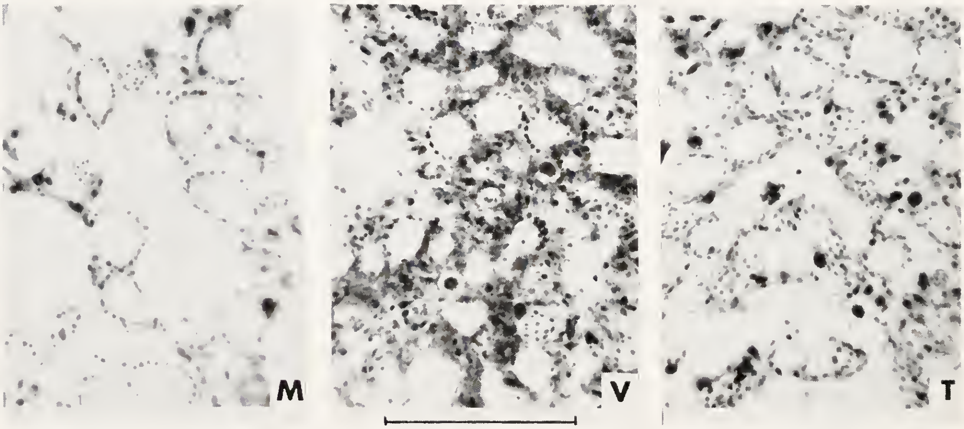

FIGURE 5. Histological sections of the three demosponges studied. Specimens have been identically processed for each species: *in situ* preservation in Bouin's fixative, 8 μ paraffin sections processed through Masson Trichrome stain, modified (Humason, 1962, page 154). (M = *Mycale* sp.; V = *Verongia gigantea;* T = *Tethya crypta;* 246 ×; scale = 100 μ).

Relationship of particle retention to anatomy

The slight differences in retention between plankton fractions (Table II, Fig. 1) and within the armored fraction (Fig. 3), may be attributable to the presence of plankton armor and to anatomical differences between sponges. Wandering amoebocytes, after picking up larger particles (> 2–5 μ in diameter) in inhalant canals, have been shown to carry out the normal pattern of digestion within phagocytic vacuoles, migrate to exhalant canals, and liberate the undigested contents of vacuoles into the post-chamber exhalant stream (van Weel, 1949; Kilian, 1964). The differences in retention rates between unarmored and armored cells is almost certainly attributable to the resistance to digestive enzymes conferred by cell armor (cellulose and siliceous tests appear to be equally resistant).

If success of digestion is furthermore a function of time of exposure, the patterns of retention within the armored fraction may be explained by differences in canal systems and tissue density between the three sponges (Fig. 5). Migration of particle-laden amoebocytes from inhalant to exhalant canal systems is expected to be rapid in *Mycale*, as physical distances are short and tissue density is low. In *Verongia*, with greater distances between canal systems and higher tissue density, migration time is expected to be extended. Amoebocytes ferrying larger particles are expected to encounter proportionally greater difficulties in *Verongia* and digestive susceptibility is expected to be a function of particle size—as found above. In *Mycale* and *Tethya*, tissue density apparently presents no appreciable obstacle to cell migration, and susceptibility to digestion in armored cells is not expected to be size-dependent—also as found above (Fig. 3).

The low retention rates of small (2 μ diameter) armored particles by *Mycale* and *Verongia* are almost certainly due to passage of large proportions of these cells through the prosopyles (2–4 μ) and flagellated chambers (between adjacent collars—not between microvilli) into the exhalant stream. The high rate of retention of these small particles by *Tethya* is not explainable by morphological differences.

Discussion

The results of this study indicate that most of the total available POC in Jamaican waters, and the dietary POC of the three sponges, consists of material retainable by glass fiber filters, but not resolvable by direct microscopy. It is not assignable to the detrital fraction as narrowly interpreted here. The existence and possible nature of this URPOC material (the deficit between CPOC and MPOC) may be inferred from close inspection of the data. Can this material be attributed to erroneous estimation of plankton fractions? Bacteria, armored cells and detrital fractions each comprise less than 2% of the total available POC. If procedural errors on carbon estimation were as high as 300 to 400% (maximal estimated for single samples) for these fractions together they could still account for only a small portion of the carbon deficit. The gross differences between retention rates of these three discrete fractions and the URPOC material increases the unlikelihood of this explanation.

Because of the inherent uncertainties in enumeration, size determination, and volume to carbon conversion of unarmored cells, only this fraction of the microscopically resolvable material could potentially contain the unaccounted for carbon. To account for the missing carbon of ambient samples in this, the largest of the resolvable fractions, the true POC content of this fraction would be at least 10 × the value calculated and more probably 50 × since these unarmored cells are expected to rupture upon contact with the filter, losing much of their carbon. The high retention rate of the unarmored fraction by all three species, however, contrasts sharply with the low rate of the missing carbon. Since procedural errors are expected to be equally applied to both ambient and exhalant samples, rates of retention of the unarmored cells and missing carbon would be expected to be equal if transformations do not take place during passage through the sponges. It is obvious, then, that if the unarmored fraction is hypothesized to contain the carbon deficit of ambient samples, it cannot at the same time account for the proportionally different deficit of exhalant samples. The unarmored fraction can contain the ambient carbon deficit only if it is further postulated that these cells are broken up or partially degraded in passing through the sponge. This would result in transformation of at least 70% of the carbon of the ambient unarmored fraction into material which is still retained by the glass fiber filters, but which is unresolvable by direct microscopy in the exhalant samples—URPOC material by definition.

The retention rates and POC calculations of each fraction thus allow only two alternative explanations for the carbon deficits: (a) the missing carbon of both ambient and exhalant samples exists as URPOC material, distinct from all resolvable fractions, or (b) the missing carbon of ambient samples is included in the unarmored fraction and is transformed in exhalant samples into material indistinguishable from the proposed URPOC material. Further information is available to aid in choosing between these alternatives. The extensive sponge fauna of the fore-reef slope habitat turns over (cycles) the 2-meter layer of water in contact with the reef each hour (from quantitative analysis of *in situ* pumping data and estimations of standing biomass of this habitat—Reiswig, unpublished). The high rate of transformation of carbon from unarmored to URPOC material postulated in alternative (b) would rapidly increase proportionate abundance of the URPOC material of ambient water. Thus the state of ambient water hypothesized in alternative (b) is dynamically unstable and rapidly would be expected to shift to the state hypothesized in alternative (a). Because alternative (b) requires assumptions of immense errors of at least an order of magnitude in calculation of unarmored POC and requires acceptance of a dynamically unstable state which appears to lead directly to the alternate assumption, the (a) hypothesis is considered to be far more likely to represent true conditions and as such has been accepted. The carbon deficit of both ambient and exhalant samples is considered to reside in a POC fraction defined as URPOC material.

The low retention of URPOC by all three sponges, and the lack of resolution of this material by light microscopy, suggests that it is quasi-particulate—*i.e.,* it is indeterminate and variable in size and shape. It is able to pass the discrete, planar filter of the choanocyte collar (0.1 μ slits) at significant rates. The effective size of URPOC is apparently within the size range of bacterial particles. Two different brands of glass fiber filters, precombusted under identical conditions, retained URPOC and bacteria at different rates. Reeve Angel #934AH filters retain 31% more total POC and 33% more of the smaller sized bacteria than the Whatman GF/C filters (means of 2 pairs of analysis), indicating that the URPOC material in these water samples has a mean effective size of approximately 0.3 μ.

On the basis of this study, the URPOC material is then hypothesized to consist of the larger size range of a continuous spectrum of quasi-particulate organic aggregates extending from isolated truly dissolved molecules to discrete detrital flakes at extremes of the range. The portion of this spectrum recognized as URPOC is defined by a lower size limit which is dependent only upon the characteristics of the glass fiber filters used for its collection—an operational and highly non-specific definition. The material is therefore hypothesized to exist in a state of physico-chemical equilibrium with truly dissolved material, from which it is generated and from which it is separated only by an arbitrary effective pore size.

Sheldon, Evelyn and Parsons (1967) have shown that organic particles are generated in standing seawater after filtration, although the formation of these particles was not proven to be independent of bacterial action. They hypothesized that the particles were bacteria, but were unable to provide incontrovertable evidence that such was the case (used the Coulter counter and plating methods, but did not make direct bacterial counts). Their particles could therefore easily be identical to the URPOC proposed here. Mullin (1965) reported that 45% of available POC of Indian Ocean waters resided in the $< 10\ \mu$ size fraction—consistent with the URPOC material proposed here. In coral reef habitats the shallow water coral-zooxanthellae communities produce a considerable excess of organic material (Kanwisher and Wainwright, 1967), probably as free polysaccharides. This may be the source material for production of URPOC in Jamaican waters. In temperate coastal regions, the products of algal communities (Bakus, 1969) may produce a similar material, although masked in these waters by the abundance of phytoplankton and resolvable detritus. The numerous reports of carbon deficits between CPOC and plankton POC (= MPOC) in marine waters throughout the world (see Jørgensen, 1966, for a review) suggest that the abundant URPOC found in Jamaican waters may reflect a general characteristic of all marine waters.

The amounts of total POC retained by these three sponges are below estimated needs for maintenance of the filtration/pumping machinery (50 mgC/m^3) and far below estimated levels required to sustain growth and reproduction (250 mgC/m^3) (Jørgensen, 1966). It will be shown elsewhere that the total natural POC demonstrated here to be retained in field populations of sponges does meet all energetic needs for two of the species—the third, *V. gigantea*, is exceptional in its requirement for dissolved organic carbon and in its possession of a vehicle for capture of DOC, symbiotic bacteria. Jørgensen's estimates are excessively in error as regards natural sponge populations, primarily because data available were inappropriate: oxygen utilization and pumping efficiencies were from very few laboratory studies, and energy conversion efficiency was taken from studies of Crustacea and Mollusca.

The results obtained here are entirely consistent with those of Pütter (1914) in which he found available plankton insufficient to satisfy dietary requirements of the sponge *Suberites massa*. Pütter hypothesized the capture and use of dissolved material by the sponge to account for the discrepancy. URPOC material, if abundant in Mediterranean waters as suggested by the differences between total POC and plankton (Sushchenya, 1963), would likewise have provided the missing carbon source and at the same time satisfy the requirements of Pütter.

In spite of the quantitative importance of URPOC in the diet of Jamaican sponges studied here, it provides little information on the particle capture systems functional in these sponges. Patterns of retention of discrete particulate fractions of the plankton, however, do indicate mechanisms of particle capture and transport. The bimodal pattern of particle retention (Figs. 1, 3 and 4) suggests that two independent systems of particle capture are functional in field populations of the three species studied here. This agrees in detail with the conclusions reached by van Trigt (1919), van Weel (1949) and others.

The primary capture system, involving particles greater than 2–5 μ, is attributable to the amoebocytes lining the inhalant aquiferous system. These cells apparently capture particles directly or phagocytose particles caught in the progressively narrower canals. This capture system must operate continuously and unselectively to prevent occlusion of the aquiferous system by suspended particles, a conclusion reached by previous workers using non-nutritive particles. The necessity for constant elimination of particles requires either a continual migrational cycling of amoebocytes from inhalant to exhalant systems, or constant physical flux of canal systems moving in space to bring excurrent canals into contact with trapped particles, or both. Evidence by van Trigt (1919), van Weel (1949), and Kilian (1952) favors the existence of amoebocyte cycling, while time-lapse cinematography of spongillids (Kilian, 1964) indicates that canal migration may be important in young sponges which have not yet developed dense populations of amoebocytes. The behavioral complexities shown by *Tethya* and *Verongia* (Reiswig, 1971) indicate that canal reorganization may be taking place in these sponges during cessation of pumping activity.

The retention differences noted between and within plankton fractions are attributable to variations of anatomy and thus to proposed differences in amoebocyte cycles, as indicated earlier (Fig. 5). More importantly, habitat restrictions are also partially explainable on this basis. *Verongia,* with a slow and easily saturated amoebocyte cycle, is expected to be less able to cope with heavy sediment loads. The species is restricted to the clean-water habitat of the outer reef. Even here it suffers serious decrease in pumping rates during increased turbidity caused by winter storms in spite of hypothesized reorganization and cleansing of canals during pumping cessation. *Mycale,* maintaining rapid amoebocyte cycles in a very loosely organized tissue reticulation, is not only able to sustain activity on the outer reef during winter storms, but is able to maintain successful populations within the sediment basin of Discovery Bay, a habitat devoid of the dense-tissue sponges.

By virtue of its loose architecture, *Mycale* appears to be able to maintain activity under a variety of conditions of turbidity, and thus invade many habitats from which more densely organized sponges are restricted, but as a corallary, a large portion of potential food as armored cells must necessarily be sacrificed via rapid particle cycling.

The situation of *Tethya* is intermediate and more complex. The diurnal cycle of pumping cessation shown by this species (Reiswig, 1971) suggests that particle capture may occur during daytime activity but digestion may extend throughout early morning cessation. The time of exposure of armored cells to digestive enzymes would be increased and utilization of the armored fraction high—as found. *Tethya* is thus able to exist in a habitat of high particle concentration, and at the same time maintain high rate of utilization of the armored cell fraction, but in this case sacrifices a significant portion of the time of filtration.

The relationships found in these three sponges between architecture and habitat restriction appear to be general for the phylum Porifera. The restriction of dense tissue Keratosa to clear water, tropical and subtropical habitats is world-wide and may be accounted for by the concomitant susceptibility of these sponges to high particle concentrations. The dominance of low tissue density sponges (*e.g.*, Haplosclerida, Poecilosclerida and Halichondrida) in temperate coastal waters with high particulate load is consistent with this scheme. It is further hypothesized that when sponges of intermediate tissue density (*e.g.*, Hadromerida) are present in coastal waters of high turbidity, behavioral complexities augmenting normal amoebocyte cleansing will be found to exist as in *Tethya*.

The secondary particle capture system, responsible for the uptake of bacteria, some of the minute 2–4 μ elements of the nannoplankton, and very probably the large URPOC fraction, consists almost certainly of the choanacytes. Capture of bacteria and bacterial-sized particles by choanocytes has been repeatedly reported by direct observation (van Trigt, 1919; Pourbaix, 1933a; van Weel, 1949; Kilian, 1952; Rasmont, 1968). Since inhalant canal systems vary between the three sponges, but the ultrastructure of the choanocyte collar is presumably uniform throughout the phylum (0.1 μ spacing between microvilli), the nearly constant rate of bacterial filtration found here almost certainly occurs at the collar surfaces. All Porifera probably remove bacteria at high efficiencies. It is interesting to note also that the immense population of symbiotic bacteria harbored by *Verongia gigantea* (and other Verongiidae—Lévi and Lévi, 1965; Vacelet, 1967) does not significantly affect net bacterial retention rate by this species. This intra- and inter-cellular population must be physically retained with high efficiency and is probably maintained at a controlled rate of population growth equal to that of the sponge.

The inability of Simpson (1963) to find extranuclear DNA in choanocytes of *Microciona prolifera* is explainable by the high density of choanocytes, the relatively low availability of bacteria per choanocyte, and the residence time of bacteria within the choanocyte. Preliminary analysis of the pumping rates of the three sponges studied here and the data of Kilian (1952) on spongillids indicates a water turnover rate of 6–20 ml/ml fresh sponge/min. Choanocyte densities of 6×10^8/ml of tissue for *Microciona prolifera* (Reiswig, unpublished) indicate a filtration rate of approximately $1.4–4.8 \times 10^{-5}$ ml/choanocyte/day. At bacterial concentrations found in Jamaica (5×10^4 cells/ml), bacterial availability is only 0.7–2.4 cells/choanocyte/day. Since residence time for particles within choanocytes is very short, probably much less than 3 hours (van Weel, 1949), accumulations of extranuclear DNA above normal background level are not expected to be detectable under natural conditions.

Efficient particle filtration in the bacterial size range, 1 μ, has been demonstrated in several other metazoan phyla (Jørgensen, 1949, 1966). The extensive studies on particle filtration in oysters, summarized in the study of particle retention in *Crassostrea virginica* by Haven and Morales-Alamo (1970), indicate that a single particle capture system is operative in these organisms. At particle sizes below 3–4 μ, retention efficiency falls rapidly in *C. virginica*, as found here in the primary amoebocyte system of sponges. In the bacterial size ranges encountered in this study 0.3–0.6 μ, retention efficiencies in *C. virginica* are less than 20%, far below the 96% level found in these sponges. The general lack of bivalves and ascidians in the outer reef habitat of Jamaica may be attributable to several factors. Species with high pumping efficiency (see Jørgensen, 1966, for tabulized summary) employ ciliary filtration (*e.g., Crassostrea*), do not utilize particles in the bacterial size range, and are probably thus unable to retain the URPOC material available in Jamaican waters. Species demonstrating high retention in the bacterial size range (*e.g., Mytilus*) employ mucous sheet filtration, and exhibit lower pumping efficiencies due to the higher "cost" of propelling water through mucous sheets. These species, although probably able to utilize significant portions of the URPOC, would be unable to meet the higher cost of lower pumping efficiency (tabulated by Jørgensen, 1966) in the relatively nutrient-poor waters of the outer coral reefs. The Porifera, strikingly specialized for efficient retention of small particles including the URPOC fraction, and exhibiting high pumping efficiencies, are apparently able to maintain their role as dominant filter feeders in coral reef situations, free of significant competition from other filter feeding taxa.

It has been shown that the choanocyte capture system supplies approximately 81.4% (URPOC + bacteria) of the total POC diet of these relatively highly organized demosponges. In less complex and presumably primitive Porifera (Calcarea, Hexactinellida, and some homoscleropborid Demospongiae) choanocytes often comprise a greater proportion of the biomass of the sponge than all other cell types. If choanocyte function is uniform, and it appears so in fresh-water and marine demosponges, the less complex sponges may be essentially restricted to the 0.1–1 μ particle size fractions. It is hypothesized that in these simple sponges little or no transfer of particles from choanocytes to amoebocytes occurs, but instead assimilation presumably takes place within individual choanocytes. Bacteria and URPOC material may thus have been the original food source of Porifera, while evolution of amoebocyte cycles later allowed the phylum to utilize larger planktonic fractions, invade temperate coastal habitats, and develop more complex, denser and larger body masses. The evidence for asconoid morphology of early Cambrian hexactinellid and heteractinid sponges (Finks, 1970) is consistent with this hypothesis. The full development of amoebocyte cycles and thick body walls has taken place almost exclusively in the Demospongiae and allowed radiation of the class throughout the continental shelves and shallow seas of the world. The generally thin-walled Hexactinellida and Calcarea, hypothesized to be almost exclusively limited to the primitive choanocyte feeding system, have presumably been unable to utilize the larger sized plankton fractions, and are thus restricted in numbers of species, form-diversity and habitat.

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SUMMARY

1. Microscopic and chemical analysis of ambient and exhalant water samples collected *in situ* indicates that the net POC diet of three tropical Demospongiae, *Mycale* sp., *Verongia gigantea* and *Tethya crypta*, consists primarily (80.5%) of

particulate (filterable) organic matter which is unresolvable by direct microscopy (URPOC). Microscopically resolvable particulate material (MPOC) accounts for only the remaining 19.5% of the POC diet of these sponges.

2. The three sponges retain available resolvable particulate material within the size range of 0.3–50 μ at high efficiencies—means are 79.0% by calculated carbon content and 82.0% by particle volume.

3. Major components of the MPOC diet and of the total POC diet of these sponges are respectively: unarmored cells 83%, 16.2%; armored cells 12.3%, 2.4%; and bacteria 4.6%, 0.9%.

4. Two functionally independent capture systems appear to be operative in all 3 species, accounting for a basic bimodal pattern of particle retention. A system involving particles between 5 and 50 μ is attributable to phagocytosis by cells lining the inhalant system. A second system involving particles of the bacterial size range (0.3–1 μ) involves capture at the choanocyte collar and ingestion by choanocytes.

5. The amoebocyte capture system is by necessity constantly functional and must accept all particles entering the ostia. A transport path by amoebocyte migration cycles is proposed to account for transport and release of intact armored plankton at significant rates.

6. Bacterial retention is high in all species, 94.8–96.9%, mean = 96.1% by cell number. This choanocyte capture system is fallible, but under normal environmental conditions retention rates are constant and independent of ambient concentrations.

7. Sponges with high tissue density (*Verongia*) show apparent size selection of armored cells which is attributed to slow amoebocyte cycling. Sponges with low tissue density (*Mycale*) retain armored fractions at lower efficiencies without apparent size selection. High retention of armored cells by *Tethya* may be attributed to behavioral adaptation.

8. All three species effect a net production of microscopically resolvable detrital organic matter.

9. The previously unrecognized unresolvable fraction of particulate organic matter (URPOC) represents an available carbon source 7 times that of all resolvable planktonic material in Jamaican waters. The ability of sponges to capture this material, probably via the primitive choanocyte system, is responsible for continuing dominance of Porifera as the filter feeders of coral reef habitats.

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SUBSTRUCTURE OF THE CORTICAL SINGLET MICROTUBULES IN SPERMATOZOA OF *MACROSTOMUM* (PLATYHELMINTHES, TURBELLARIA) AS REVEALED BY NEGATIVE STAINING[1]

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The cortical singlet microtubules of the spermatozoa of certain flatworms have been circumstantially implicated in motility. The motile spermatozoa of *Plagiostomum* have cortical singlet microtubules and no axonemes (Christensen, 1961), and the bodies of the spermatozoa of *Dugesia* and *Bdelloura* (Silveira and Porter, 1965) and *Mesostoma* (Henley, Costello, Thomas and Newton, 1969) contain singlet microtubules and undulate independently of the two free flagella.

Following negative staining, the cortical singlets of the spermatozoa of some species of flatworms, namely the lungfluke *Haematoloechus* (Burton, 1966a, 1966b, 1970) and the polyclads *Stylochus* (Thomas, 1970) and *Notoplana* (Henley, in press), have a helical wall structure. There have been observed in these forms transitions to a protofibrillar configuration, which is the more typical configuration of the subunits in some negatively stained singlets (Gall, 1966), doublets (André and Thiéry, 1963; Grimstone and Klug, 1966; Pease, 1963) and triplets (Wolfe, 1970).

Because of the molecular dimorphism of the microtubules and their probable function in the motile process, cortical singlet microtubules are of interest as a model system for microtubule-associated motility. Therefore, knowledge of the size, number, and arrangement of the subunits making up the walls of the microtubules is important to understanding their function.

The number of subunits which occurs around the circumference of microtubules has been established for some species (for a review, see Arnott and Smith, 1969), but this is not the case for the cortical singlets of spermatozoa of the flatworms. From the results of rotational analysis, Burton (1966a) has suggested that there are 8 subunits around the circumference of the cortical singlet microtubules of the spermatozoa of *Haematoloechus*. Negative staining reveals the presence of 6 or 7 protofibrils for the cortical singlets of the spermatozoa of *Stylochus* (Thomas, 1970). However, the number of protofibrils seen in negatively stained preparations probably represents only a portion of the entire complex in most cases. This may be due to maceration of some of the microtubules (see Henley, 1970) or to superimposition of one half of the protofibrils on the other half (Burton, 1966b).

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The motile spermatozoon of *Macrostomum* contains cortical singlet microtubules and there are no free or incorporated axonemes (Fig. 1). Negative staining reveals that, typically, the subunits of the cortical singlets are helically arranged, and sometimes undergo a transition to the protofibrillar configuration; this latter configuration was found to terminate in at least 12 protofibrils for a number of singlet microtubules.

MATERIALS AND METHODS

Specimens of *Macrostomum* sp. were collected from still water below the dam of University Lake near Chapel Hill, North Carolina. Whole animals were placed in a Columbia watchglass containing 1% aqueous phosphotungstic acid (PTA), pH 6.8. The animals were teased with steel needles, and drops of the PTA, containing spermatozoa freed from the animal, were transferred to Formvar-carbon-coated 200-mesh copper grids. After approximately 6 minutes, the fluid was drained from the grids which were then allowed to dry; preparations were examined with the Zeiss 9A electron microscope.

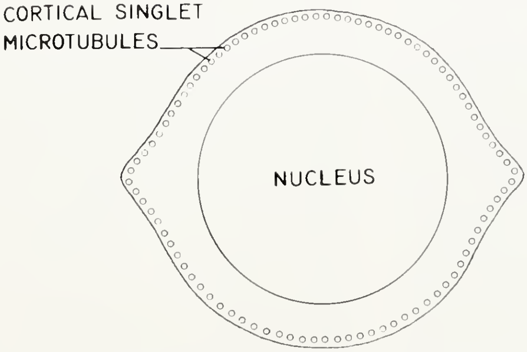
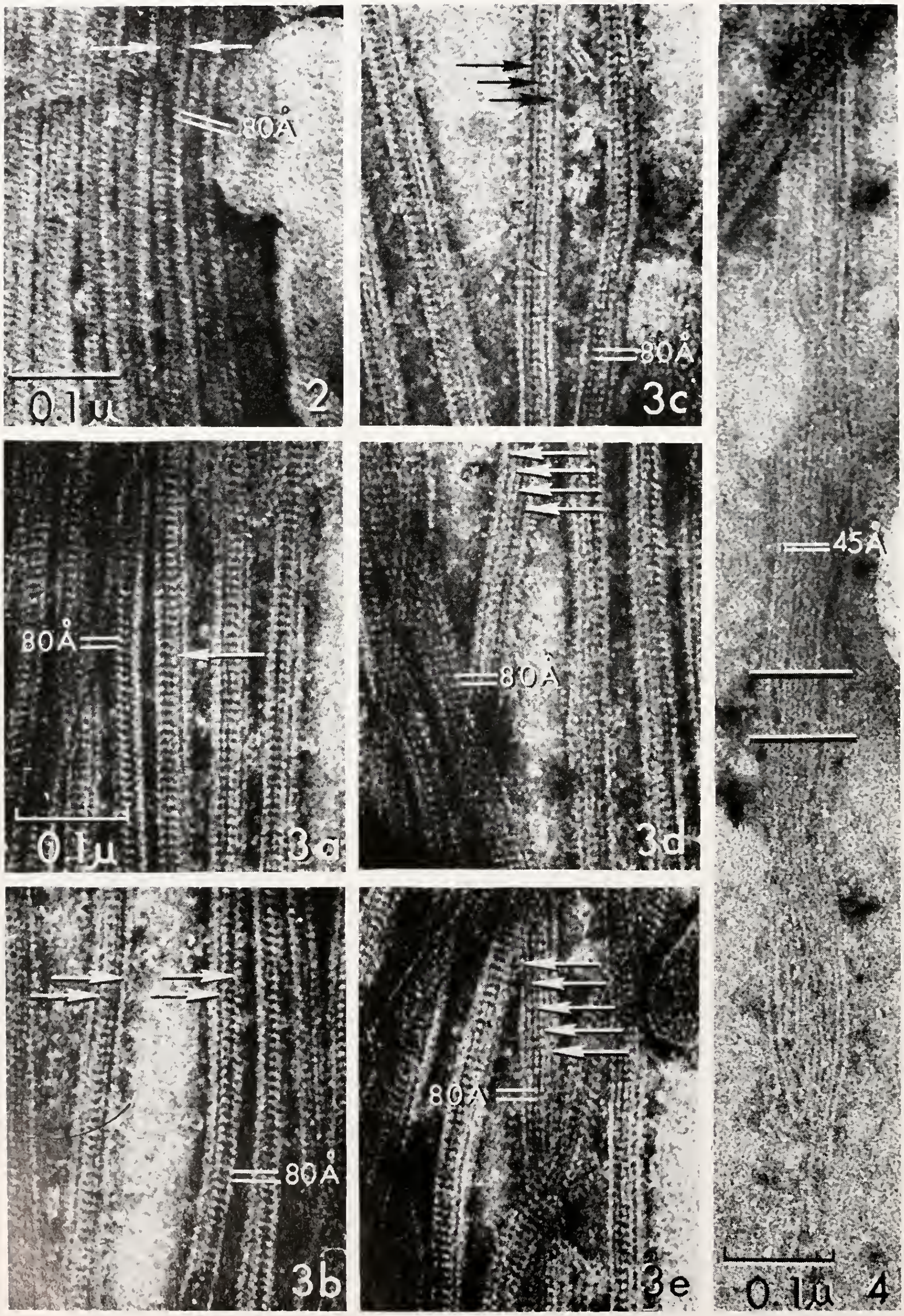


FIGURE 1. Diagram of the arrangement of cortical singlet microtubules in the nuclear region of a spermatozoon of *Macrostomum*. No axonemes are present.

RESULTS

In sectioned spermatozoa of *Macrostomum*, there are *ca.* 60–80 cortical singlet microtubules; 30–40 were present in material negatively stained with PTA (the remainder presumably having been digested away or lost spatially from the total complement during the process of negative staining). Three arrangements of the subunits in these PTA-treated microtubules were found. (1) In a few regions, the subunits were in the intact helical configuration (Fig. 2), comparable to that described for cortical singlet microtubules in spermatozoa of other flatworms. (2) There were large areas in which the lateral spacing of the subunits was increased, but the circumferential integrity of the tubules appeared to be preserved in such a way that only the top halves of the microtubules could be seen (Fig. 3a-e). (3) At their termini, these microtubules ended in protofibrils lying in one plane (Fig. 4).


80Å
0.1μ
2
80Å
3c
45Å
80Å
0.1μ
3a
80Å
3d
80Å
3b
80Å
3e
0.1μ
4

Figures 2–4.

(1) The width of the microtubules having the subunits arranged in the most compact helical configuration (Fig. 2) is *ca.* 200–210 Å; the alternating electron-lucent and electron-dense bands have a center-to-center spacing of *ca.* 80–85 Å and are inclined at an apparent angle of *ca.* 12–14°. The subunits appear to be closely apposed along the helical path, for there is no line of demarcation between adjacent subunits. Accumulation of the electron-dense PTA along the central axis suggests the presence of a lumen, indicating that the microtubule is cylindrical or only slightly flattened. Measurement of the cortical singlets in sectioned material likewise indicates a diameter of *ca.* 200 Å.

(2) Varying degrees of lateral separation of adjacent subunits occur in other regions. In those showing the least change from the helical configuration (Fig. 3a), one protofibrillar element is separated laterally from the remainder of the microtubule, which retains its helical arrangement of subunits. The overall width of the microtubule here is *ca.* 260 Å and there is a *ca.* 50 Å gap between the separated protofibril and the remainder of the microtubule; the helical portion is approximately 180 Å wide.

The opposite extreme of separation of the subunits is shown in Figure 3e, in which there are 6 protofibrils with a lateral center-to-center spacing of 50–60 Å. The overall width of the microtubule has increased here to 370 Å.

Intermediate stages between the two extremes are numerous and four examples are shown in Figures 3b-e, where arrows indicate two, three, four and five lateral separations, respectively. Analysis of the intermediate stages indicates that whereas the lateral spacing between protofibrils varies from 50 to 70 Å, the center-to-center spacing between subunits along the length of the protofibrils is much more constant, with the 80 Å spacing characteristic of the helical configuration being maintained, as noted on the figures.

(3) The third arrangement of subunits is in the protofibrillar configuration (Fig. 4). This differs significantly from the protofibrillar arrangement described above (2) following lateral separation of the protofibrils, and also from the protofibrillar configuration described for cortical singlets in spermatozoa of other flatworms. The maximum number of protofibrils previously reported for negatively stained cortical singlets is 6 or 7 (Thomas, 1970), but in Figure 4, a complement of 12 protofibrils can be seen for a single microtubule. The overall width of the ribbon of protofibrils here is *ca.* 650 Å and the minimum lateral separation of the protofibrils is 45–50 Å. Furthermore, in contrast to the 80 Å longitudinal center-to-center spacing of subunits along the separated portions of the microtubules as described in (2) above, the longitudinal spacing here is 40–45 Å.

FIGURE 2. Intact cortical singlet microtubules (arrows) of a spermatozoon of *Macrostomum*, showing the compact helical configuration of subunits typical of the cortical singlets of some of the platyhelminths; magnification: 152,000 ×.

FIGURE 3. Cortical singlet microtubules of a spermatozoon of *Macrostomum*, showing examples of progressive stages in the lateral separation of the protofibrils. Figures 3a, b, c, d, and e show one, two, three, four and five lateral separations, respectively. In each figure arrows indicate the region of the microtubule representative of the stage. Other patterns can often be seen in adjacent regions of the same microtubule and in adjacent microtubules; magnification: 152,000 ×.

FIGURE 4. Cortical singlet microtubules of a spermatozoon of *Macrostomum* in which the subunits are in the protofibrillar configuration. Twelve protofibrils can be counted in the region between the two lines; magnification: 152,000 ×.

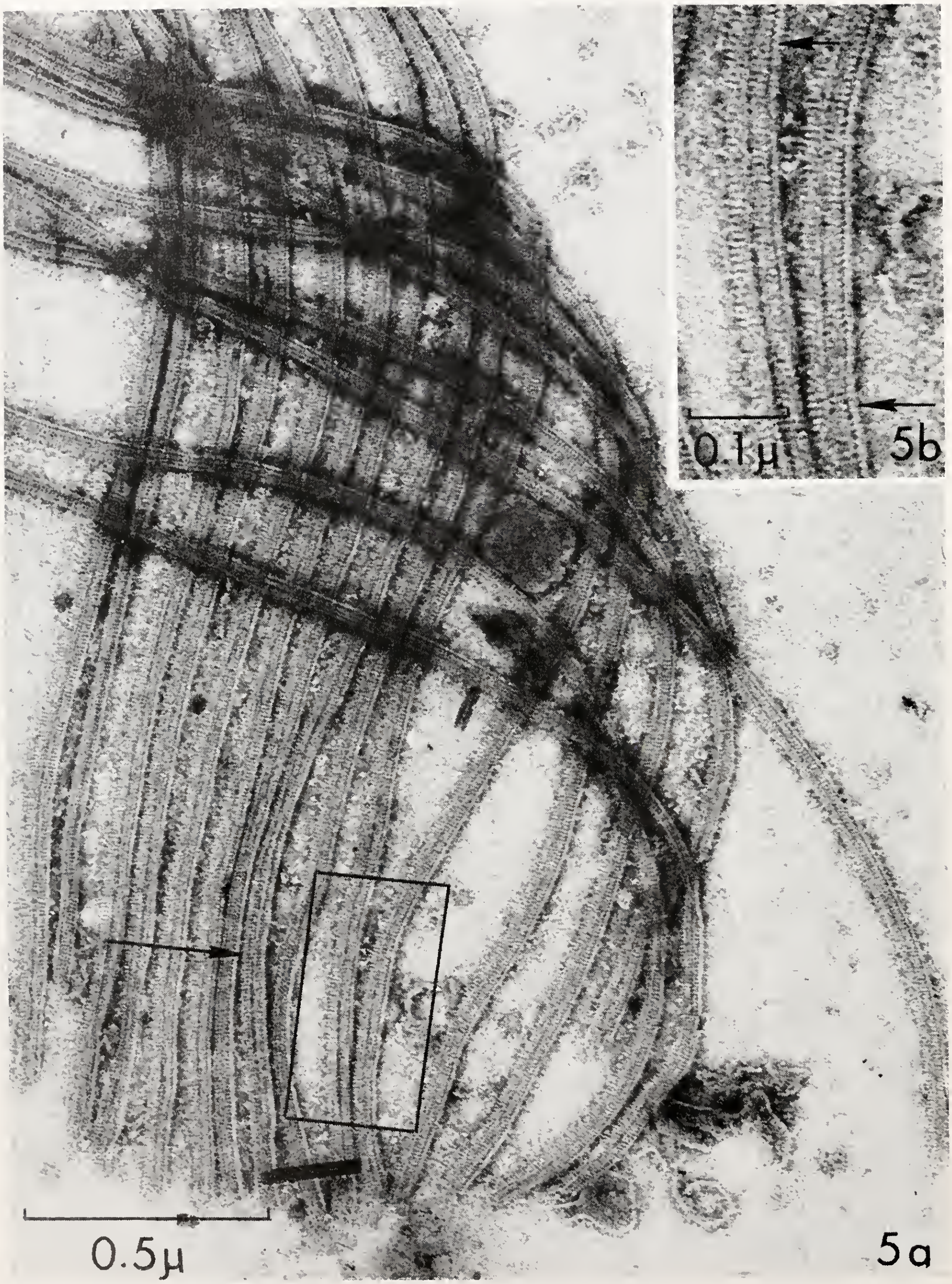


FIGURE 5a. Terminal portions of 17 cortical singlet microtubules. The appearance of two helical structures for each microtubule could result if the top half of each microtubule slid off the bottom half while the microtubule was still in the helical configuration. The arrow indicates the region in which the width is *ca.* 460 Å. A single protofibril is associated with the right half-microtubule in each case; magnification: 62,700 ×.

Figures 5a and 5b show regions in which the top halves of the microtubules have slid off the bottom halves, the subunits remaining in the helical configuration. Superficially observed, the microtubules here appear to branch into two (Fig. 5a, arrow) microtubules; however, there is no accumulation of the negative stain along the central longitudinal axis of each "subtubule," and therefore no indication of the presence of a lumen. The width of the "double" structure at the point indicated by the arrow is *ca.* 460 Å. The width following 5 lateral separations (Fig. 3e) was *ca.* 370 Å, and for one lateral separation the overall width was usually *ca.* 260 Å. In Figure 5a and adjacent micrographs (not shown) of the entire complement of microtubules, each of the 17 "paired" structures can be traced to 17 intact singlet microtubules. Of interest is the observation that in all 17 microtubules shown in Figure 5a, a single protofibril, of slightly less electron density than the remainder of the structure, is invariably located on the same side of all 17 microtubules (Fig. 5b, arrows). The significance of this observation is not known.

DISCUSSION

It has been suggested that the cortical singlet microtubules of the spermatozoa of *Plagiostomum* (Christensen, 1961), *Dugesia* and *Bdelloura* (Silveira and Porter, 1965) and *Mesotoma* (Henley *et al.*, 1969) are involved in motility. The cortical singlets from spermatozoa of *Haematolocchus* (Burton, 1966a, 1966b), *Stylochus* (Thomas, 1970) and *Notoplana* (Henley, in press) have been shown to have a helical arrangement of subunits. *Macrostomum* is the only species described thus far in which it is known that the spermatozoa are motile, with cortical singlets as their only microtubular component and with the subunits of the singlets helically arranged.

The transitions thus far described in the literature, from the helical to the protofibrillar configuration in cortical singlet microtubules, are usually quite abrupt (Burton, 1966a, 1966b; Thomas, 1970). In the cortical singlets described here, however, many stages in the transition are evident, providing a unique opportunity for understanding the event. This variability is undoubtedly the consequence, at least in part, of the macerating action of PTA (Henley, 1970), which makes feasible a study of the transitions in their various manifestations. Analysis of the changes which occur in the transition from the helical to the protofibrillar configuration suggests that two separate events occur. One change involves a lateral separation of the subunits to produce *ca.* 12 protofibrils, with a variable lateral spacing of 50–70 Å and a longitudinal center-to-center spacing of *ca.* 80 Å of subunits along the protofibril. The separation of any two adjacent protofibrils appears to occur at random, as evidenced by the wide variety of patterns shown in Figures 3a–e, and diagrammed in Figure 6. The diagram is based on the possible occurrence of 12 protofibrils, with the top six visible. Similar variability can, of course, be obtained with other numbers, but the observed range of patterns can be interpreted only with 11, 12 or 13 protofibrils.

The second change is in the spacing of the subunits along the length of a protofibril. In the helical configuration the subunits are spaced *ca.* 80 Å center-to-

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FIGURE 5b. Enlargement of the area outlined by the rectangle in Figure 5a, showing the two helical portions and associated single protofibrils (arrows); magnification: 152,000 ×.

center along the length of the microtubule. This 80 Å spacing is maintained during lateral separation. However, in a truly protofibrillar configuration, such as that shown in Figure 4, the center-to-center spacing of the subunits is 40–45 Å. The possibility that this is a visual effect, produced by superimposition of protofibrils, is negated by the fact that the protofibrils are lying in parallel. Although

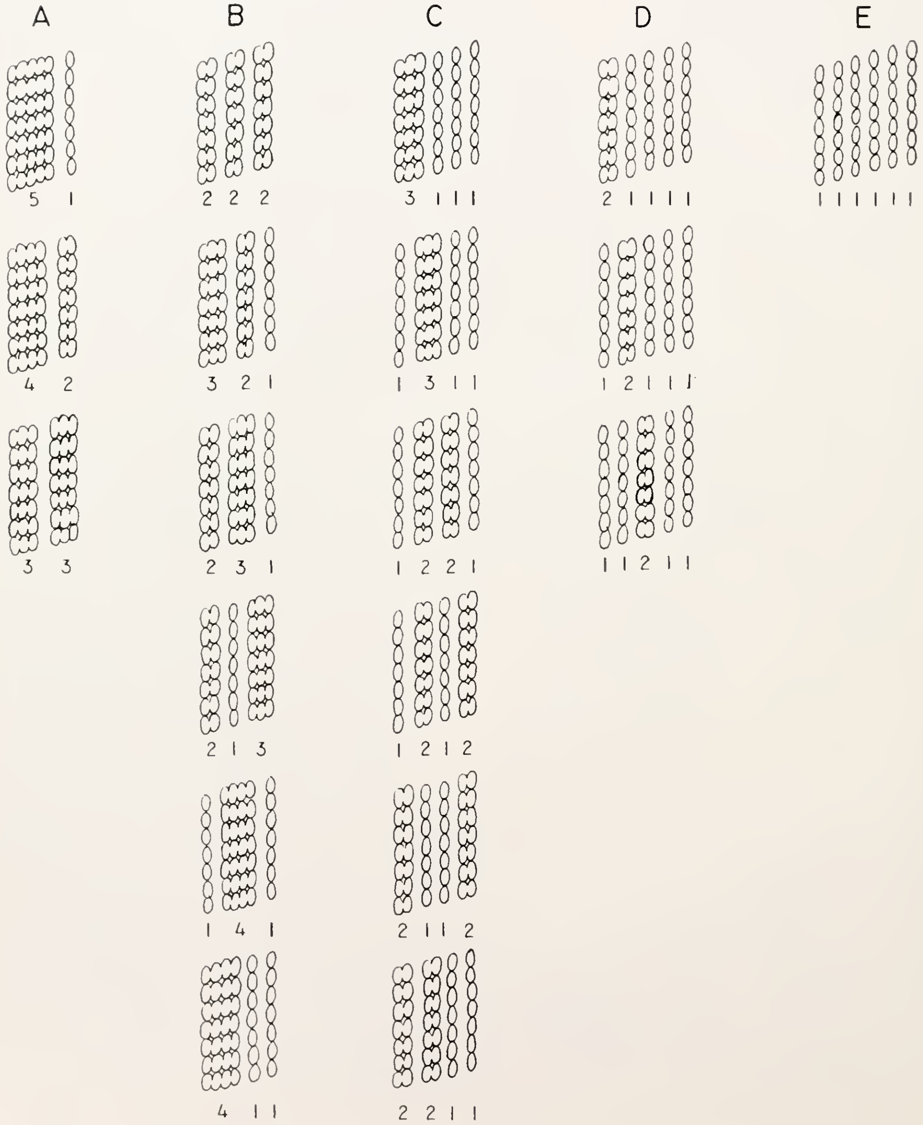
A
B
C
D
E
5 1
4 2
3 3
2 2 2
3 2 1
2 3 1
2 1 3
1 4 1
4 1 1
3 1 1 1
1 3 1 1
1 2 2 1
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FIGURE 6. Diagram illustrating the possible array of patterns that could be exhibited by a microtubule if there are 12 protofibrils, of which the top 6 are visible, and if lateral separation of protofibrils occurs at random. Mirror images are not illustrated.

lateral separation can occur without a concomitant change in the longitudinal spacing, the change from 80 Å to 40–45 Å has never been observed by us to occur in the absence of lateral separation.

The shape and associations of the repeating units along the length of the intact microtubules in the helical configuration are not known. There are at least three possible models that could explain the presence of the alternating electron-lucent and electron-opaque bands which repeat at intervals of *ca.* 80 Å along the length of the microtubule (Fig. 7A). One such alternative is based on the assumption that the walls of the microtubules are made up of ovoid subunits which are *ca.* 80 Å in length and joined end to end along the length of the protofibrils (Fig. 7B). In the region in which contact between adjacent subunits occurs, a slight

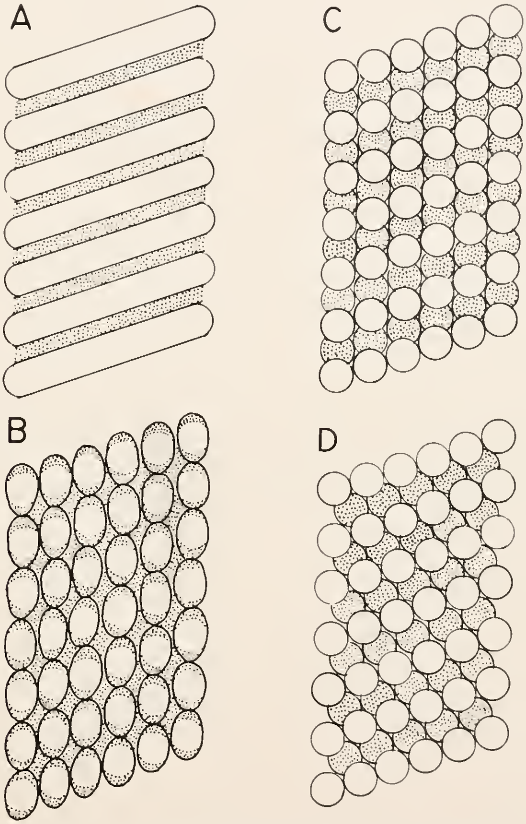

FIGURE 7. (A) Diagram of an intact cortical singlet microtubule as it appears following negative staining with PTA. The center-to-center spacing between adjacent white bands in this, as in (B), (C) and (D), is 80 Å; (B) Helically arranged ovoid subunits, joined end to end along the length of the protofibrils, as they might appear following negative staining; (C) and (D) Helically arranged globular subunits with alternating subunits slightly displaced toward the lumen of the microtubule, either parallel to the long axis of the microtubule (C) or nested among the outer subunits (D).

depression or groove would be formed in which the electron-opaque negative stain would accumulate, thereby producing alternating light and dark bands with an 80 Å repeat. The observed shift in spacing between subunits along the long axis of the microtubule, from the 80 Å periodicity in the intact microtubule (Fig. 2) to the 40–45 Å spacing in the truly protofibrillar configuration (Fig. 4), is in keeping with such a model of longitudinally oriented, elongated subunits, if a dimer-monomer relation were involved as well as a conformational change.

Two other models take into account the shift in spacing between subunits along the long axis of the microtubule. In one such model, alternate subunits along a protofibril are recessed slightly (about 10% of their diameters) toward the lumen of the microtubule (Fig. 7C). The recessed subunits could lie parallel to the protofibril (Fig. 7C) or nested among the outer subunits (Fig. 7D). In either case there would be formed around the microtubule a groove which would accumulate the electron-dense negative stain and produce the alternating bands with a repeat of 80 Å. A more widely spaced arrangement of outer subunits than those portrayed in Figures 7C and 7D is required here, since the recessed subunits would make a cylinder of smaller diameter.

There is biochemical evidence that the shift in spacing of subunits, from 80 Å in the intact microtubule to 40–45 Å in the protofibrillar configuration, may involve dissociation of dimers to form monomers along the length of the protofibrils. Shelanski and Taylor (1968) have reported that the subunits of microtubules in sea urchin sperm flagella can be isolated as dimers with a molecular weight of *ca.* 120,000 (and a corresponding particle size of 40–50 × 80–90 Å), or as monomers with a molecular weight of *ca.* 60,000 (and a particle size of 40 Å). If their biochemical results reflect the existence of dimers and monomers in the intact microtubule, then it is of interest to relate the occurrence of such configurations to the results observed here. The random nature of the lateral separation of subunits described gives no indication that monomers associate into dimers along the helical path (at an oblique angle to the long axis of the microtubule). However, the described shift in spacing of the subunits along the length of the protofibrils may be suggestive of an association of monomers to form dimers along the length of the protofibrils. Within such a framework, the formation of the groove to produce the alternating bands in the intact microtubule might be explained. If two monomers form an ovoid dimer with the long axis parallel to the long axis of the microtubule, the appearance might be similar to that shown in Figure 7B. Similarly, if one half of the dimer is inclined toward the lumen of the microtubule, the groove might be formed in a manner similar to that shown in Figures 7C and 7D. It is possible that freeze-etch or shadow-casting may give some information as to the contours of the subunits. Such experiments are in progress and may be useful in deciding among the possible alternatives.

SUMMARY

1. Cortical singlet microtubules are the only microtubular components of the motile spermatozoa of *Macrostomum*. When negatively stained with phosphotungstic acid, the microtubules display a helical arrangement of the subunits similar to that described for spermatozoa of other species of platyhelminths.

2. The protofibrillar configuration of subunits of the cortical singlets can also occur under conditions of negative staining. One microtubule was clearly observed to be made up of 12 protofibrils.

3. Analysis of various stages in the transition from the helical to the protofibrillar configuration suggests that there are two steps in the conversion. These are (a) a seemingly random lateral separation of subunits to form protofibrils with a longitudinal periodicity of *ca.* 80 Å, which is characteristic of the periodicity of the intact helical arrangement, and (b) a subsequent change in spacing of the subunits along the length of the protofibril, from *ca.* 80 Å to 40–45 Å. These observations support the view that if monomers associate to form dimers, the dimers occur along the length of the protofibril rather than between adjacent protofibrils.

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